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THE ANEMIA OF RENAL INSUFFICIENCY AS INDUCED BY BILATERAL NEPHRECTOMY OF THE RABBIT: WITH EMPHASIS ON ITS HEMOLYTIC NATURE

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OBSERVATIONS of the effect of bilateral nephrectomy of dogs during the past four years from this institution have demonstrated the development not only of hypertension and cardiovascular lesions, but also of a severe anemia, which required correction in order to extend the survival of the animals.[1] The spleen, the mesenteric lymph nodes, and the liver of these animals displayed hemosiderosis and erythrophagocytosis, thereby suggesting a hemolytic process of intracellular type.[1, 2] The present communication, previously presented in abstract form,[3] describes studies on rabbits after bilateral nephrectomy which attempt to identify the extent, mechanism, and characteristics of this anemia.

METHODS

Young adult white rabbits, of either sex, weighing 2 to 3.5 kg., were used. These animals were offered Purina rabbit chow and water ad libitum before and during the experimental period.

Operative Technique.—Under ether anesthesia, the kidney was approached retroperitoneally by an incision caudal to the costal margin. The kidney was delivered through a small incision (about 2 inches), and the pedicle was ligated. The procedure was conducted with a negligible degree of blood loss. The second nephrectomy was performed about one week after the first in order to minimize operative trauma. Postoperatively, 25,000 units of penicillin were injected subcutaneously.

The control blood volume and other measurements were determined one week following the first nephrectomy. The results of the determinations on this sample were compared with the results taken three days after the removal of the second kidney. At this time the animal was sacrificed and the gastrointestinal contents were collected for the pigmentary studies. In addition, specimens from the spleen and liver were obtained for the determination of the iron concentration of these organs.

Ten rabbits acted as controls for all procedures. The following manipulations were performed on these animals. The same operative technique was used for these animals as for the test animals. One kidney was approached retroperitoneally and delivered externally, the perirenal fat was removed, and the renal pedicle was cleared in the usual manner preparatory to its ligation. The kidney was then returned to its bed, and the wound was sutured. One week

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later the same procedure was conducted on the other kidney. In this manner the animal and both kidneys were subjected to the same procedures as the test animals with the exception of the ligation of the renal pedicle and removal of the kidneys. The blood volume and other determinations were performed one week following the first renal manipulation and three days after the second manipulation. At this time the animal was sacrificed, and the gastrointestinal contents were collected for the determination of its total content of biliary pigment. Also samples of spleen and liver were obtained for the determination of the iron concentration of these organs.

Blood Volume, Red Blood Cell Mass, and Plasma Volume.—The blood volume was determined with P^{32} as described by Reid and Orr[4] and with the blue dye T-1824, as described by Mayerson, Lyons, Parson, Nieset, and Trautman.[5] The hematocrit was determined by Wintrobe's method.[6] Heparin was used as the anticoagulant. With the dye method, a correction factor of 0.92 was used for the hematocrit in order to align it more closely with the total body hematocrit.[5] The red blood cells tagged with P^{32} were obtained from healthy donor rabbits. The measured quantity of red cells tagged with P^{32} (0.2 ml. including the buffer, with approximate count of 14,000) and the amount of T-1824 dye (1.0 mg. in 1.0 ml.) were pipetted into a 5 ml. syringe and mixed. The mixture was injected into the marginal ear vein of the rabbit, and the syringe was rinsed twice with 2 ml. of normal saline, the washings being injected through the same needle. A single sample of 4 ml. for the determination of the concentration of T-1824, radioactivity, and the other measurements was obtained by cardiac puncture with a 20-gauge needle, resulting in a minimal or no hemopericardium was revealed at autopsy.

Mixing Time for Volume Determinations.—As suggested by Gregersen,[7] one sample immediately following the established mixing time for the dye was taken. The validity of this procedure for the rabbit has been confirmed.[8] The optimal mixing time for the rabbit, with the use of cardiac blood, was determined to be 12.5 minutes, and this time was used for all determinations. Extrapolation from the disappearance slope gave results similar to the 12.5-minute results, as discussed by three group of workers.[5, 7, 8]

Apparent State of the Circulation.—At the time of the blood volume determination the state of the circulation was considered adequate by the following criteria: the seemingly steady state of the animal, the regular heart action, the normal tonicity of the skeletal musculature, and the rapid and proper filling of the marginal ear vein after milking it empty. Moribund animals were not tested; as a consequence thirty-five animals were nephrectomized in order to obtain data on ten.

Effect of the Operation on the Blood Volume.—The effect of a single nephrectomy on the blood volume determination was tested by determinations before and after the operation. That the operation per se did not cause anemia is demonstrated by the data of Table I, which contains the results for the red blood cell volume by the two methods used.

The total iron content of all stool specimens was determined as a means of estimating the blood loss via the bowel. By considering hypothetically that all of this iron was derived

TABLE I. RED CELL VOLUME BEFORE AND AFTER NEPHRECTOMY
(NOT SAME AS TEST ANIMALS)

ANIMAL	P^{32} METHOD (ML.)		T-1824 METHOD (ML.)	
	BEFORE	AFTER	BEFORE	AFTER
1	26.5	26.7	27.6	24.9
2	37.5	36.5	39.1	38.3
3	38.1	36.2	36.5	36.6
4	35.7	35.9	34.9	29.4
5	34.5	34.8	31.4	29.8
6	31.5	34.1	31.2	33.1
7	34.5	28.4	35.6	27.9
8	32.0	30.0	32.9	29.4
9	26.5	26.6	24.4	26.3
10	30.3	30.4	29.4	29.2
Average	32.7	31.9	32.3	30.5

TABLE II

ANIMAL	TIME, DAYS	ANIMAL WEIGHT (KG.)	HEMATOCRIT (PER CENT)	T-1824 METHOD			P^{32} METHOD		
				TOTAL BLOOD VOLUME (ML.)	PLASMA VOLUME (ML.)	RED CELL VOLUME (ML.)	TOTAL BLOOD VOLUME (ML.)	PLASMA VOLUME (ML.)	RED CELL VOLUME (ML.)
Test Animals									
1	0	2.56	43.0	221	126	95	230	131	99
	3	2.61	34.8	119	78	41	125	82	43
2	0	2.93	41.9	355	206	149	359	209	150
	3	2.98	40.3	160	96	64	173	104	69
3	0	2.73	32.9	238	160	78	240	161	79
	3	2.61	30.2	125	87	38	119	83	36
4	0	2.44	34.3	184	121	63	179	118	61
	3	2.33	25.2	142	106	36	146	109	37
5	0	2.24	38.4	182	112	70	194	120	74
	3	2.33	29.7	153	107	46	148	104	44
	4	2.27	13.9	178	153	25	176	152	24
6	0	2.50	35.7	153	98	55	160	103	57
	3	2.39	33.9	129	85	44	127	84	43
7	0	2.39	39.1	151	92	59	156	95	61
	3	2.39	38.0	120	74	46	119	74	45
	4	2.39	36.0	110	70	40	106	68	38
8	0	2.50	36.7	143	91	52	158	102	56
	3	2.39	30.1	102	71	31	103	72	31
9	0	2.50	41.3	171	100	71	183	107	76
	3	2.39	30.1	118	82	36	113	79	34
10	0	2.39	42.5	153	88	65	161	93	68
	3	2.20	28.1	103	74	29	111	80	31
Mean values	0	2.52	38.6	195	119	76	202	124	78
	3	2.46	32.1	127	86	41	134	88	46
Control Animals									
1	0	1.99	31.4	167	115	52	169	116	53
	3	1.99	32.0	160	109	51	158	107	51
2	0	2.27	29.8	138	96	42	136	95	41
	3	2.27	30.9	150	104	46	153	106	47
3	0	2.73	31.7	130	89	41	131	89	42
	3	2.67	30.0	141	99	42	142	99	43
4	0	2.94	33.1	142	96	46	138	92	46
	3	2.84	32.9	154	103	51	149	100	49
5	0	2.27	37.3	143	90	53	147	92	55
	3	2.27	38.5	140	86	54	150	92	58
6	0	2.22	41.3	147	86	61	151	90	61
	3	2.16	41.6	157	92	65	161	94	67
7	0	2.39	44.1	160	89	71	154	86	68
	3	2.27	46.3	158	85	73	156	84	72
8	0	2.16	35.8	154	99	55	166	107	59
	3	2.16	35.3	159	103	56	168	109	59
9	0	2.61	39.6	143	87	56	152	92	60
	3	2.44	39.0	141	86	55	154	94	60
10	0	2.94	31.8	158	108	50	155	106	49
	3	2.84	32.8	152	102	50	156	105	51
Mean values	0	2.45	35.6	148	96	53	150	97	53
	3	2.39	35.9	151	97	54	155	99	56

0 indicates the values taken prior to the second nephrectomy; 3 indicates the values three days after the second nephrectomy; 4 values four days after the second nephrectomy.

from hemoglobin, the possibility of gastrointestinal hemorrhage as a cause of the anemia was controlled. The total amount of iron in the stool collections and gastrointestinal contents at autopsy was negligible.

Determination of the Biliary Pigment in the Stools.—The biliary pigment of the stools was determined by three methods. The urobilinogen content was determined by the method of Watson, Schwartz, Sborov, and Bertie.[9] In addition, the determination of total stoool pigment was conducted by the methods of Malloy and Evelyn[10] and the method employed by Sibhishaj, Hawkins, and Whipple.[11] The later methods have been considered to include all bile pigments. Three-day collections of stools were obtained before the first nephrectomy (intact animal), after the first and after the second nephrectomy. The total stool was weighed and the biliary pigment was determined in an aliquot of a sample after homogenization in a Waring blender. The results were expressed as the total stool pigment excreted per day. The same collection periods were used for the control animals with reference to the manipulations of the kidneys. In addition the total content of biliary pigment of the gastrointestinal contents, as collected at the time of the autopsy, was determined. The control animals acted as reference for the nephrectomized animals.

The blood urea was determined by direct nesslerization according to Taylor and Blair.[12] The method of Folin and Wu[13] was used for the blood NPN concentration. The method of Malloy and Evelyn[14] was used for the bilirubin determinations. The serum iron was determined by the method of Barkam and Wallace.[15] The iron-binding capacity of the serum was obtained by the technique used by Rath and Finch.[16] The percentage saturation of iron in the serum represented a ratio of these two values. Samples of liver and spleen obtained at autopsy were dried to constant weight in an oven, and the total iron content was measured by the method of Scott.[17] The same method was used for the stool iron content. The reticulocyte,[18] platelet,[19] and white blood cell[18] counts were conducted by standard procedures. The mechanical fragility of the erythrocytes was determined by a modification of the method described by Ham, Castle, Gardner, and Daland.[20] A plastic disc, 8 inches in diameter, rotating thirty times per minute, held test tubes 75 by 10 mm. in size at right angles to the circumference. One ml. of blood plus two beads (4 mm. in diameter) were placed in the tube, rotated for ninety minutes following which the per cent of total hemolysis was determined.

Bone marrow from the innominate bone was touched on glass slides and stained with Wright-Giemsa stain. The differential count was compared with samples from normal rabbits.

RESULTS

The data obtained from the nephrectomized and the control animals are tabulated in Tables II, III, IVA, IVB, and V.

Blood Urea and NPN (Table III).—Azotemia was pronounced, since in these experiments no attempt was made to lower the level of nitrogenous products by means of extrarenal measures.

Hematocrit (Table II).—The hematocrit reading was lowered, but the reduction of the reading did not reflect the extent of the anemia as revealed by the blood volume studies.

Red Blood Cell Volume (Table II).—All animals revealed comparable reductions of the red blood cell volume, as determined by the two methods. The reduction in the red blood cell volume, within three days, varied between minus 20 and minus 57 per cent of the preazotemic value, the mean value was minus 41 per cent. Two animals which were retested on the fourth day, revealed additional reductions.

The rapid decline in red blood cell volume, without blood loss, indicated not only a rapidly developing anemia, but the presence of hemolytic process. Although the quantitative accuracy of these measures has been questioned in

TABLE III

ANIMAL	TIME (DAYS)	UREA BLOOD (MG./100 ML.)	NPN MG./100 ML.	SATURATION LIMIT FOR SERUM IRON (μG/100 ML.)	SERUM IRON (μG/100 ML.)	IRON SATURATION (PER CENT)	DIRECT SERUM BILIRUBIN (MG./100 ML.)	INDIRECT SERUM BILIRUBIN (MG./100 ML.)	TOTAL SERUM BILIRUBIN (MG./100 ML.)	MECHANICAL FRAGILITY OF R.B.C. (PER CENT TOTAL HEMOLYSIS)
Test Animals										
1	0	37	40	275	82	29.8	Neg.	0.11	0.11	3.9
	3	346	199	458	208	45.4	0.13	1.32	1.45	14.8
2	0	33	47	209	97	46.4	Neg.	0.17	0.17	3.7
	3	246	162	508	288	56.7	0.42	1.72	2.14	13.3
3	0	50	47	255	67	26.3	Neg.	0.68	0.68	4.0
	3	224	158	488	224	45.9	1.08	3.64	4.72	8.9
4	0	41	38	276	101	36.6	Neg.	0.17	0.17	3.9
	3	364	197	521	341	65.5	0.87	3.04	3.91	9.9
5	0	39	42	287	87	30.3	Neg.	0.37	0.37	5.9
	3	371	212	448	198	43.8	1.24	2.48	3.72	8.4
	4	407	243	488	228	46.7	1.88	4.63	6.51	13.9
6	0	43	39	305	100	32.8	Neg.	0.43	0.43	4.2
	3	184	108	456	206	45.2	1.24	3.03	4.27	7.8
7	0	36	40	299	89	29.4	Neg.	0.18	0.18	4.6
	3	204	156	467	217	46.3	1.14	1.87	3.01	9.5
	4	281	198	534	274	51.2	2.36	2.43	4.79	11.6
8	0	34	30	334	134	40.1	Neg.	0.16	0.16	5.6
	3	270	161	449	224	50.0	1.00	2.01	3.01	8.1
9	0	28	26	306	106	34.6	Neg.	0.84	0.84	3.6
	3	314	188	488	263	53.8	1.36	2.87	4.23	6.4
10	0	27	31	308	108	35.0	Neg.	0.86	0.86	4.6
	3	351	191	526	296	56.3	1.86	3.61	5.47	8.0
Mean values	0	37	38	285	97	34.1	Neg.	0.40	0.40	4.4
	3	268	183	481	246	51.0	1.03	2.56	3.59	9.5
Control Animals										
1	0	41	44	325	120	36.8	Neg.	0.27	0.27	5.2
	3	36	42	321	113	35.2	Neg.	0.23	0.23	5.8
2	0	42	39	319	119	37.3	Neg.	0.53	0.53	3.4
	3	54	42	332	129	38.8	Neg.	0.58	0.58	4.5
3	0	47	31	331	131	39.6	Neg.	0.33	0.33	6.1
	3	59	36	314	120	38.2	Neg.	0.33	0.33	7.6
4	0	39	40	288	100	34.8	Neg.	0.41	0.41	5.3
	3	42	38	287	97	33.8	Neg.	0.36	0.36	5.6
5	0	32	30	281	91	32.4	Neg.	0.24	0.24	3.1
	3	30	27	276	76	27.6	Neg.	0.16	0.16	3.7
6	0	38	32	303	98	32.3	Neg.	0.32	0.32	2.6
	3	36	38	310	110	35.5	Neg.	0.63	0.63	5.4
7	0	24	30	307	112	36.4	Neg.	0.36	0.36	5.6
	3	30	27	289	89	32.5	Neg.	0.39	0.39	6.5
8	0	31	29	311	106	34.1	Neg.	0.84	0.84	8.9
	3	32	33	310	98	31.6	Neg.	0.79	0.79	7.9
9	0	33	31	330	130	39.4	Neg.	0.69	0.69	8.9
	3	36	30	326	121	37.1	Neg.	0.84	0.84	7.6
10	0	42	37	301	86	28.6	Neg.	0.38	0.38	4.9
	3	44	33	317	97	30.6	Neg.	0.91	0.91	4.8
Mean values	0	37	34	310	119	35.2	Neg.	0.44	0.44	5.4
	3	40	35	308	105	34.1	Neg.	0.52	0.52	5.9

TABLE IV A

ANIMALS	TIME (DAYS)	STOOL UROBILINOGEN (WATSON METHOD) (UNITS/DAY)	STOOL BILE (WHIPPLE METHOD) (MG. BILIRUBIN/DAY)	STOOL BILE (MALLOY METHOD) (MG. BILIRUBIN/DAY)	STOOL IRON (μG/DAY)	IRON CONCENTRATION LIVER (μG/GM. DRY TISSUE)	IRON CONCENTRATION SPLEEN (μG/GM. DRY TISSUE)
			Test Animals				
1	−3 to 0	580	54	66	10		
	0 to 3	1612	269	278	60		
	Autopsy	1062	361	307	103	394	4002
2	−3 to 0	671	49	43	15		
	0 to 3	1225	209	196	56		
	Autopsy	1192	313	364	63	463	3900
3	−6 to −3	693	43	51	33		
	−3 to 0	584	47	43	41		
	0 to 3	1921	189	206	89		
	Autopsy	1483	286	293	106	497	3042
4	−3 to 0	494	47	38	31		
	0 to 3	1251	126	101	64		
	Autopsy	1955	264	284	188	---	----
5	−6 to −3	534	57	61	17		
	−3 to 0	686	61	67	20		
	0 to 3	2790	291	312	64		
	3 to 4	2397	414	404	101		
	Autopsy	1578	507	536	137	621	4152
6	−6 to −3	536	56	66	18		
	−3 to 0	578	64	62	18		
	0 to 3	1283	136	129	33		
	Autopsy	2143	198	207	120	542	3170
7	−6 to −3	512	52	55	15		
	−3 to 0	508	48	55	17		
	0 to 3	1248	128	121	37		
	3 to 4	2374	278	250	80		
	Autopsy	1830	304	317	129	845	2370
8	−6 to −3	534	49	60	15		
	−3 to 0	431	46	51	13		
	0 to 3	1130	138	158	58		
	Autopsy	1426	214	201	82	436	2881
9	−6 to −3	581	37	42	15		
	−3 to 0	458	41	44	16		
	0 to 3	1312	169	163	65		
	Autopsy	1241	188	176	77	444	6354
10	−6 to −3	410	44	52	16		
	−3 to 0	358	39	40	14		
	0 to 3	1194	175	181	63		
	Autopsy	1136	169	174	70	462	3250
Mean	−6 to −3	554	49	53	19		
values	−3 to 0	515	49	52	20		
	0 to 3	1496	183	184	59		
	Autopsy	1505	281	286	108	523	3680

−6 to −3 indicates the three-day interval prior to the first nephrectomy or renal manipulation. −3 to 0 represents the three-day interval after the first nephrectomy or manipulation. 0 to 3 indicates the three-day interval after the second nephrectomy or manipulation. "Autopsy" indicates the total gastrointestinal contents obtained post mortem.

the literature, they may be considered as indicative of the trend and rapidity of change in the red blood cell mass. The significance of the data is believed to be confirmed by the other hematologic abnormalities described later.

TABLE IV B

ANIMALS	TIME (DAYS)	STOOL UROBILINOGEN (WATSON METHOD) (UNITS/DAY)	STOOL BILE (WHIPPLE METHOD) (MG. BILIRUBIN/DAY)	STOOL BILE (MALLOY METHOD) (MG. BILIRUBIN/DAY)	STOOL IRON (μG/DAY)	IRON CONCENTRATION LIVER μG/GM. DRY TISSUE	IRON CONCENTRATION SPLEEN (μG/GM. DRY TISSUE
			Control Animals				
1	−6 to −3	525	51	58	25		
	−3 to 0	366	48	46	17		
	0 to 3	441	53	54	14		
	Autopsy	218	87	73	14	239	682
2	−6 to −3	473	43	49	21		
	−3 to 0	388	47	53	20		
	0 to 3	402	31	47	15		
	Autopsy	300	53	62	16	301	714
3	−6 to −3	364	41	56	13		
	−3 to 0	470	50	49	13		
	0 to 3	501	39	43	13		
	Autopsy	230	98	83	15	188	436
4	−6 to −3	514	62	59	12		
	−3 to 0	498	48	52	11		
	0 to 3	306	54	63	11		
	Autopsy	241	77	87	10	214	504
5	−6 to −3	370	34	39	11		
	−3 to 0	352	41	38	8		
	0 to 3	404	38	42	9		
	Autopsy	194	63	70	9	304	623
6	−6 to −3	540	38	41	17		
	−3 to 0	497	42	39	17		
	0 to 3	506	46	52	16		
	Autopsy	216	64	69	10	198	389
7	−6 to −3	424	53	47	23		
	−3 to 0	396	50	52	18		
	0 to 3	425	47	41	16		
	Autopsy	221	73	82	17	208	541
8	−6 to −3	508	59	50	19		
	−3 to 0	434	61	60	18		
	0 to 3	494	54	51	16		
	Autopsy	188	123	136	16	311	582
9	−6 to −3	374	35	40	12		
	−3 to 0	389	39	36	12		
	0 to 3	306	46	51	13		
	Autopsy	194	131	129	14	283	478
10	−6 to −3	414	39	41	11		
	−3 to 0	473	52	57	11		
	0 to 3	406	47	54	11		
	Autopsy	201	89	94	8	243	593
Mean values	−6 to −3	451	46	48	16		
	−3 to 0	426	48	49	15		
	0 to 3	419	46	50	13		
	Autopsy	220	80	89	13	249	554

Total Blood Volume (Table II).—The blood volume, as determined by the T-1824-hematocrit and the P^{32} methods, was also reduced, but not to the same extent as the red blood cell volume. The reduction of the total blood volume varied between minus 15 and minus 55 per cent of the preazotemic value (mean minus 28 per cent).

Plasma Volume (Table II).—The plasma volume either determined more directly by the dye method or indirectly by the P^{32} method, indicated the least tendency to diminish. The reduction varied between minus 5 and minus 53 per cent (mean minus 25 per cent).

Bile pigment in the stool (Tables IVA and IVB). The quantity of bile pigment in the stool per day by all three methods was significantly increased over the preazotemic values. Moreover, the amount of total bile pigment in the gastrointestinal contents at autopsy of the nephrectomized animals was significantly greater than that of the control animals.

Serum Bilirubin (Table III).—The total serum bilirubin was elevated in all animals. The direct reacting bilirubin was not detectable either in the control animals or in the prenephrectomy samples, but all animals revealed its presence after bilateral nephrectomy. Although the concentration of direct bilirubin was not prominent (0.13 to 2.36 mg. per 100 ml), the fact that the change in direct bilirubin was of an all or none type is considered significant. The indirect bilirubin was elevated in all rabbits, varying between 1.32 and 4.63 mg. per 100 ml. The magnitude of the jaundice thus varied, the total serum bilirubin fluctuating between 1.5 and 6.5 mg. per 100 ml. The mild jaundice was commensurate with the high excretion of bile pigment in the stool.

Serum Iron (Table III).—The serum iron was elevated in all animals. The increment varied between 67 and 237 per cent (mean 156 per cent) of the level prior to the renal insufficiency.

Saturation Limit for Serum Iron (Table III).—The serum saturation limit for binding iron, that is the total available iron-binding globulin, was elevated in all rabbits. The increment varied between 34 and 143 per cent of the control value.

Percentage Saturation of Serum Iron (Table III).—The extent of the saturation of the iron-binding globulin of the serum was elevated between 24 and 87 per cent of the control figure.

The results of the iron determinations indicate a hyperferremia associated with an increment in the total iron-binding globulin of the serum. The serum iron, however, was increased to a greater extent than the iron-binding globulin, consequently the percentage saturation was not as prominently elevated as the serum iron.

Tissue Iron (Tables IV A and IV B).—The iron content of the spleen and liver included the residual blood within these tissues. The results have been compared with similar observations on the ten control rabbits. The iron content of both tissues was increased over the control values, but the increment in the spleen was much greater than that for the liver.

Red blood cell count and peripheral hemoglobin concentration. (Table V). The red blood count and hemoglobin concentration were moderately depressed, the decrement being of similar magnitude as that of the hematocrit reading. Computation of the MCV, MCH, and MCHC demonstrated that the anemia was of normochromic, normocytic type.

TABLE V

ANIMAL	TIME (DAYS)	W.B.C. PER CU. MM.	R.B.C. PER CU. MM.	HEMOGLOBIN (GM./100 ML.)	PLATELETS PER CU. MM.	RETICULOCYTES (PER CENT)
			Test Animals			
1	0	9,250	6,500,000	14.2	150,000	2.3
	3	8,020	4,360,000	10.4	120,000	3.4
2	0	9,700	6,200,000	12.5	120,000	0.5
	3	11,300	5,100,000	9.4	250,000	2.00
3	0	13,050	5,240,000	11.5	241,040	5.4
	3	11,050	4,930,000	10.0	403,260	2.3
4	0	9,100	5,550,000	12.5	190,000	0.5
	3	10,000	4,300,000	10.4	150,000	2.4
5	0	8,050	5,250,000	11.5	180,000	0.1
	3	7,900	4,800,000	9.2	120,000	0.5
	4	8,200	4,100,000	7.8	120,000	0.4
6	0	7,250	4,150,000	10.8	150,000	0.5
	3	8,400	3,850,000	8.6	160,000	0.8
7	0	9,150	5,250,000	12.3	200,000	0.0
	3	7,450	4,150,000	9.0	180,000	2.6
	4	8,200	3,900,000	8.2	150,000	3.6
8	0	9,550	5,350,000	10.8	430,000	3.7
	3	7,200	4,180,000	8.2	230,000	4.6
9	0	9,450	5,100,000	12.8	190,000	2.0
	3	8,120	3,850,000	9.6	150,000	1.8
10	0	7,850	5,050,000	12.6	140,000	2.0
	3	8,600	3,400,000	9.4	120,000	4.5
Mean values	0	8,240	5,364,000	12.2	159,100	1.7
	3	8,804	4,292,000	9.4	138,300	2.5
			Control Animals			
1	0	9,200	3,310,000	8.5	130,000	0.1
	3	11,350	3,200,000	8.4	120,000	0.2
2	0	6,400	3,430,000	8.0	180,000	0.5
	3	5,500	3,780,000	9.2	160,000	0.4
3	0	8,800	3,410,000	8.7	270,000	0.6
	3	8,100	4,130,000	8.0	130,000	0.8
4	0	9,550	3,860,000	8.0	150,000	0.5
	3	10,450	4,270,000	8.5	200,000	0.6
5	0	9,250	3,100,000	8.7	190,000	1.0
	3	9,150	3,310,000	8.9	180,000	1.2
6	0	9,700	4,050,000	12.3	190,000	4.2
	3	11,050	3,900,000	11.9	160,000	3.8
7	0	9,150	3,860,000	10.8	140,000	1.6
	3	8,050	3,600,000	11.0	180,000	1.6
8	0	10,050	4,400,000	11.3	180,000	2.5
	3	8,800	4,150,000	10.2	140,000	2.5
9	0	8,340	3,950,000	11.9	200,000	2.4
	3	6,650	4,400,000	11.9	150,000	3.5
10	0	6,400	4,450,000	9.5	190,000	2.6
	3	5,500	4,350,000	10.8	200,000	2.5
Mean values	0	8,684	3,782,000	9.8	182,000	1.6
	3	8,460	3,909,000	9.9	162,000	1.7

Leukocyte, Platelet, and Reticulocyte Counts (Table V).—The white blood cells and platelets were counted in order to determine whether these two formed elements were affected along with the erythrocytes. After the second nephrectomy, these values remained essentially unaltered. The reticulocyte count also remained unaltered.

Iron Content of the Stools (Tables IV A and IV B).—The iron content of the stools was increased after the second nephrectomy. The quantity, however, was negligible when translated into the corresponding volume of red blood cells. Thus, blood loss via the bowel did not account for the anemia.

The mechanical fragility of the erythrocytes during the uremic state was elevated about 2 by 3 times over the preazotemic level (Table III).

The weights of the animals remained steady throughout the period of observation (Table II).

Examination of smears taken from the bone marrow of the innominate bone of five animals after bilateral nephrectomy revealed a cellular marrow with a normal or increased percentage of normoblasts.

Control Rabbits.—As revealed in the various tables, all determinations on the control rabbits, subjected to the renal manipulations but not to the nephrectomies, remained steady. Thus the changes noted following the removal of the second kidney are considered significant.

DISCUSSION

These studies have indicated the rapid development of anemia following bilateral nephrectomy of the rabbit associated with a rapidly developing and extreme azotemia. This anemia was indicated by a fall in the hematocrit and a decrement in the red blood cell volume as revealed by the T-1824 dye-hematocrit and P^{32}-hematocrit methods. The plasma volume was reduced to a lesser extent than the red blood cell volume. Although the quantitative accuracy of these methods is open to question, their usage as an index of the directional change, and within limits, of the extent of the change in the red blood cell mass appears warranted. The significance of these volume estimates is strengthened by certain collateral hematologic observations.

The rapid recession of the red blood cell mass in the absence of blood loss indicates that a hemolytic process was concerned with the production of the anemia. The elevations of the serum bilirubin and the biliary pigment in the stool indicated the presence of hemolysis. The changes in the serum iron concentration may also be construed as indicative of hemolysis, for it has been demonstrated that hemolytic anemias during activity are associated with an elevated serum iron concentration and an elevated saturation limit for iron in the serum.[21] The bone marrow of five animals displayed a normal or elevated percentage of normoblastic elements. Finally, the increment in the content of iron in the spleen was of such magnitude as to indicate an accelerated breakdown of red blood cells. It appears evident, therefore, that the anemia was of the hemolytic type.

The hemolytic system involved in the observed hemolysis has not been elucidated, although the frequent observation of erythrophagocytosis within tissues rich in reticuloendothelial elements in the dog under similar circumstances, suggest strongly a phagocytic or intracellular form of red blood cell destruction. The greater degree of siderosis in the spleen in the rabbit, as well as in the dog, represents an additional feature supporting this view. Finch

and co-workers[22] have demonstrated a greater concentration of iron in the spleen in hemolytic states. Whether some degree of intravascular destruction also occurred was not tested.

Although the anemia was associated with a profound azotemia, it remains to be demonstrated whether an adequate removal of the nitrogenous waste products after bilateral nephrectomy is associated with a prevention of the anemia. There are indications that this may be true, for recently it has been noted that dogs whose blood urea concentration was maintained at lower azotemic levels by means of peritoneal irrigation did not reflect the same magnitude of anemia as those animals with a high blood urea level. The correlation of anemia with azotemia does not necessarily indicate that nitrogenous waste represent the damaging agents to erythrocytes.

The hemolysis was not associated with reticulocytosis. The relative reticulocytopenia indicated the simultaneous occurrence of suppression of the bone marrow and the failure of acceleration of the delivery of young red blood cells to the circulation. Suppression of bone marrow function, however, must be relegated to a secondary role in view of the rapidity of development of the anemia.

It is of interest that total renal insufficiency due to renal ablation did not depress the peripheral concentration of leukocytes and platelets, suggesting that the peripheral mechanism is selective for the erythrocytes.

The demonstration that bilateral nephrectomy of experimental animals, and its attendant azotemia, are associated with a hemolytic mechanism of rapid action, may be of practical interest. The well-known occurrence of anemia in human malignant hypertension has one experimental counterpart in the hypertension and anemia in animals after renal ablation. Recently certain observations in patients add to this interest. Callen and Limarzi[23] have demonstrated the occurrence of normal or hyperplastic cellularity of the bone marrow in 80 per cent of patients with the anemia of azotemia. This indicates that the cellular population of the bone marrow per se is not at fault. Emerson[24] has presented evidence indicating the occurrence of hemolysis in a patient with renal failure and azotemia due to glomerulonephritis. Loge, Lange, and Moore[25] have shown by means of red blood cell survival studies that certain patients with renal failure and anemia destroy infused red blood cells at a rate substantially greater than normal. Finch, Gibson, Peacock and Fluharty[26] have shown a depressed uptake of radioactive iron by the peripheral blood of patients with azotemia, indicating a suppression of bone marrow function.

It cannot be stated that the mechanisms in these patients and those in the rabbit and dog are similar, but a similarity becomes more likely by these experimental and clinical observations.

SUMMARY

Bilateral nephrectomy of rabbits has been shown to be associated with the rapid development of anemia. The anemia was identified by a lowering of the hematocrit reading, erythrocyte count, hemoglobin concentration, and decrease

in the red blood cell volume as indicated by the T-1824 dye and the radioactive phosphorus methods. That the anemia was not due to blood loss was demonstrated by the low iron content of the stools and the absence of hemorrhage at autopsy. The rapidity of development of the anemia was construed as indicating a hemolytic process, and conversely, precluded the possibility of its resulting from a dietary deficiency. Additional evidence supporting a hemolytic state included elevation of the serum bilirubin and stool urobilinogen, elevation of the serum iron concentration and the saturation limit of the serum for iron, a normal or elevated erythroid concentration in the bone marrow, and an increase in the iron content of the spleen. Evidence for an intracellular type of hemolysis had been formerly noted in the dog by the simultaneous demonstration of hemosiderosis and erythrophagocytosis. In the rabbit intracellular hemolysis would be in keeping with the data obtained. A relative reticulocytopenia indicated the concomitant occurrence of suppression of bone marrow function for red blood cells. The production of leukocytes and platelets was apparently unaltered.

Certain recent observations on the anemia of uremia in patients indicate the occurrence of hemolysis in some patients. A hemolytic component in the anemia of malignant hypertension has not been determined. However, the occurrence of anemia in malignant hypertension and anemia and hypertension in experimental animals after bilateral nephrectomy is interesting.

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DIURESIS PRODUCED BY CONCENTRATED DIALYZED HUMAN AND PREGNANT MARE URINE. AN ASSAY PROCEDURE IN THE DOG FOR DIURETIC SUBSTANCES

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IN A previous paper[1] it was reported that the intravenous infusion of normal human and dog urines and dialyzed human urine into normal unanesthetized dogs, in volumes equivalent to 0.5 to 1.0 per cent of the body weight, resulted in a marked increase in the rate of urine flow. The diuresis represented a reduction in total body water of the animal equivalent to approximately 1.0 to 1.5 per cent of the body weight. These earlier experiments were complicated by the necessity of infusing an appreciable volume of fluid, and also by the necessity of infusing artificially constituted control solutions.

In order to extend this study, it was necessary to perfect the assay technique. Some factors which affect the excretion of a water load were previously reported,[2] but further study showed an occasional false diuretic response in control experiments. Two additional aspects of the excretion of a fluid load by dogs have been studied: (a) a comparison of the urinary recovery following oral loading with 0.9 per cent sodium chloride solution and tap water, and (b) the variability of urinary excretion during three-time intervals following such loads. Based on these studies a revised assay technique is reported here. The reports of Adolph and others[3] have been utilized freely in these studies.

The study has been extended, using the revised technique, to include concentrated dialyzed human urine, concentrated dialyzed human urine which had been lyophilized, and concentrated dialyzed pregnant mare urine which had been lyophilized. These studies are reported here. All of these preparations result in a significant diuresis, i.e., a loss of body water in relation to the control state of the animal. For comparison, the diuresis produced by Thiomerin (mercaptomerin sodium) under these conditions was determined.

MATERIALS AND METHODS

Preparation of the Animal for Load Recovery Experiments.—Female dogs weighing 6 to 12 kg. were prepared for catheterization by an episiotomy, and they were trained to lie quietly on the animal board with loose restraining thongs. At 4:00 P.M. on the day before the experiment, the animals were given 25 ml. per kilogram of tap water by stomach tube as a hydrating dose, and the food was removed. Water was left available and was removed at 8:30 A.M. on the day of assay. At 10:00 A.M., on that day, the animals were catheterized, the bladder was emptied, and the urine was discarded, the catheter being left in place. At the same time, a 0.9 per cent NaCl solution load of 25 ml. per kilogram or a tap water load of 25 ml. per kilogram was given by stomach tube. At

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11:00 A.M. the first collection period was ended. At 12 noon the second collection period was ended and the animals were returned to metabolimeter cages without food or water. At 4:00 P.M. the bladders were emptied by catheterization ending collection period three (see Table I). The animals were kept in a room maintained at 22° C., except for the first two hours of the assay period.

Assay Procedure for Diuretic Substances.—The time schedule given above was adopted. At 10:00 A.M. the animals received an oral tap water load of 25 ml. per kilogram. At 11:00 A.M., at the end of the first collection period, the substance being studied was administered, or, if it was a control experiment, 2 ml. of distilled water was injected intravenously. The total urine volume excreted during hours 2 through 6 was used to compare the effect of a diuretic substance with the control state (see Table II).

Urine Preparations: The human urine specimens were collected in sterile bottles under conditions approaching asepsis as nearly as possible, and all dialyses were carried out in the refrigerator in order to minimize bacterial growth. Each preparation represented a pool of urine collected from male laboratory workers, without evidence of disease, the collection being the early morning voiding in each case. These specimens were transferred to sterilized dialysis bags, and they were dialyzed for forty-eight hours against running tap water and for twenty-four hours against running distilled water. The urine was concentrated by evaporation from the dialysis bags facilitated by blowing air across the bags at room temperature. Sufficient evaporation usually required about fifteen hours. The final volume of the concentrate was adjusted to represent a thirty-fold concentration of the original volume. The dry weight of the pool was determined on an aliquot, and the remainder was frozen and kept frozen until it was used for assay. Thawing was accomplished by immersion in water at about 35° C.

The lyophilized* human urine specimen was prepared by dissolving 14 Gm. in 100 ml. of sterile water. Dialysis was carried out as above, and since the specimen was cloudy it was filtered before being concentrated to 25 ml. as above.

The lyophilized* pregnant mare urine was prepared by dissolving 20 Gm. in 400 ml. of sterile distilled water. It was dialyzed as above. A sediment formed during concentration; the supernatant fluid was removed and made to a volume of 50 ml. with sterile distilled water.

All urine preparations were administered to the dogs by the intravenous injection of 0.4 ml. per kilogram.

RESULTS

Recovery of Saline or Water Load.—In Table IA will be found the recovery of a load of 25 ml. per kilogram of 0.9 per cent NaCl solution given by stomach tube. The coefficients of variation for the first hour after administration of the load are quite large, the average being 50.7 per cent, while the coefficients of variation for hours 2 through 6 and hours 3 through 6 were about equal and somewhat smaller. The average recovery of the load during six hours was 68.7 per cent.

In Table IB also will be found the recovery of a load of 25 ml. per kilogram of tap water given by stomach tube. The average coefficient of variation for the first hour and for hours 3 through 6 are approximately the same as for salt solution. However, the average coefficient of variation for hours 2 through 6 is relatively low, 17.6 per cent, and is considerably lower than the value for hours 3 through 6. The average recovery of the load during six hours was 93.6 per cent.

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TABLE I. URINARY VOLUME FOLLOWING A FLUID LOAD GIVEN BY STOMACH TUBE. EIGHT EXPERIMENTS ON EACH DOG

DOG. NO.	FIRST HOUR: MEAN VOL. (A) (ML./KG.)	FIRST HOUR: σ	FIRST HOUR: C.V. (PER CENT)	HOURS 2 THROUGH 6: MEAN VOL. (B) (ML./KG.)	HOURS 2 THROUGH 6: σ	HOURS 2 THROUGH 6: C.V. (PER CENT)	HOURS 3 THROUGH 6: MEAN VOL. (C) (ML./KG.)	HOURS 3 THROUGH 6: σ	HOURS 3 THROUGH 6: C.V. (PER CENT)	TOTAL RECOVERY IN 6 HOURS: SUM OF MEANS A + B	TOTAL RECOVERY IN 6 HOURS: PER CENT OF LOAD
(A). LOAD: 25 ML./KG. OF 0.9% NACL SOLUTION											
21	3.0	1.52	50.8	16.6	3.60	21.7	13.2	2.22	16.9	19.6	78.5
22	7.8	3.45	44.3	14.4	3.61	25.1	8.9	2.33	26.2	22.2	88.8
24	1.5	0.52	34.7	7.3	2.57	35.2	6.1	2.66	27.1	8.8	35.2
25	2.1	1.49	71.0	13.1	4.45	34.0	9.8	2.57	26.2	15.2	60.8
26	1.6	0.46	28.7	9.8	5.89	60.1	9.3	7.52	80.8	11.4	45.6
27	5.5	3.07	55.9	14.2	4.42	31.1	8.6	4.15	48.3	19.7	78.8
28	4.2	1.58	37.7	14.1	4.16	29.6	10.2	3.33	32.7	18.3	73.2
29	2.1	1.41	67.1	10.3	5.03	48.8	8.2	4.47	54.5	12.4	49.6
30	3.7	2.63	71.1	12.8	3.64	28.4	7.5	1.80	24.0	16.5	66.0
31	4.5	1.35	30.0	13.7	2.70	19.7	9.9	2.21	22.3	18.2	72.8
32	9.5	6.27	66.0	17.1	4.27	24.9	10.1	2.15	21.3	26.6	106.3
Average			50.7			32.6			34.6		68.7
Range			28.7–71.1			19.7–60.1			16.9–80.8		
(B). LOAD: 25 ML./KG. OF TAP WATER											
21	7.0	5.67	81.0	13.1	2.35	17.9	5.4	1.65	30.4	20.1	80.4
22	12.8	2.59	20.2	17.2	4.44	25.8	8.6	4.57	53.2	30.0	120.0
24	2.8	1.29	46.1	8.4	1.70	20.2	4.9	2.00	40.8	11.2	44.8
25	7.6	6.47	85.1	20.6	2.95	14.3	15.1	3.52	23.3	28.2	112.8
27	9.2	3.28	35.7	13.1	2.27	17.3	4.5	1.34	31.4	22.3	89.3
28	5.6	2.83	50.6	14.3	1.78	12.5	6.3	1.24	19.7	19.9	79.7
29	5.2	3.10	59.6	14.5	1.92	13.2	6.8	2.43	35.8	19.7	78.8
30	11.0	3.65	33.2	15.7	2.97	18.9	5.2	2.54	48.9	26.7	106.8
31	8.5	2.53	29.8	15.6	2.82	18.1	7.8	2.86	36.7	24.1	96.5
32	11.4	4.74	41.6	16.4	2.92	17.8	5.3	2.37	44.7	27.8	111.2
33	11.0	4.84	44.0	16.4	2.96	18.0	2.5	2.07	82.8	27.4	109.7
Average			47.9			17.6			40.7		93.6
Range			20.2–85.1			12.5–25.8			19.7–82.2		

σ = standard deviation; c.v. = coefficient of variation.

TABLE II. VOLUME OF URINE EXCRETED FOLLOWING THE INTRAVENOUS INJECTION OF PREPARATIONS OF DIALYZED CONCENTRATED HUMAN URINE, PREGNANT MARE URINE, AND THIOMERIN (MERCAPTOMERIN SODIUM). INDIVIDUAL OBSERVATIONS WITH EACH PREPARATION

PREPARATION	DRY WEIGHT	DOG NUMBER AND VOLUME EXCRETED IN ML./KG./5 HOURS									
		21	22	25	27	28	29	30	31	32	33
CONTROL MEAN ¶		13.1	17.2	20.6	13.1	14.3	14.5	15.7	15.6	16.4	16.4
σ		2.35	4.44	2.95	2.27	1.78	1.92	2.97	2.82	2.92	2.96
MEAN ± 2σ	MG./ML.	8.4-17.8	8.3-26.1	14.7-26.5	8.6-17.6	10.7-17.9	10.7-18.3	9.8-21.6	10.0-21.2	10.6-22.2	10.5-22.3
1*	0.6	21.7	39.5	35.6	20.7	29.8	26.2	36.5	29.5	25.2	22.9
2	1.5				26.0	18.4	31.6	26.9	28.3		
3	1.5	32.1	31.7	43.8	35.7	27.6	33.2	28.9	31.1	30.9	34.7
4	3.6	34.5	38.8	56.5						27.5	22.7
5	4.4	20.9	33.6	26.8						25.4	32.9
6‖	1.1				22.3 / 25.1 \	23.0	27.3	*19.0* / 26.5 \	30.0		
7	3.5	23.2	*26.0*	35.8	31.8	30.2	33.7	26.9		23.4	36.2
8†	2.8	27.6	*22.6*	*23.0*	19.4	*17.2*	24.6	25.2		24.1	31.6
9a	0.8				37.6	19.5	23.0	38.6	30.0		
9b†, ‡	0.8	20.0	*25.4*	*22.5*	*13.1*		18.5	31.3		*20.8*	23.2
10†	2.1	*14.6*	*20.0*	14.4§	19.4	27.0	32.2	23.9	43.7	*16.4*	*16.8*
11†	2.6	19.0	*13.6*	*15.5*	*17.1*	21.3	25.1	26.4	32.8	*16.4*	23.7
Thiomerin A		19.5	34.1	27.8	22.3	22.3	29.2	*17.8*	21.6	*19.3*	23.9
Thiomerin B		28.7	63.7	*20.6*	19.4	37.7	32.7	22.2	27.9	23.6	27.1

Values lying within the range control mean ± 2 σ are italicized and are considered to be nonsignificant variations from the control mean.
Preparations 1 through 8 are human urine preparations.
Urine preparation dose = 0.4 ml./kg.
Thiomerin A dose = 0.3 ml./kg. of a 1:10 dilution of the commercial preparation, equivalent to 1.2 mg./kg. Hg.
Thiomerin B dose = 0.5 ml./kg. of preparation A, equivalent to 2.0 mg./kg. Hg.
*Lyophilized human urine.
†Lyophilized pregnant mare urine.
‡This is preparation 9a after 74 days storage in the frozen state.
§This is the only significant antidiuretic response seen.
‖The same preparation was given on separate days to two of the dogs.
¶These data are transferred from Table IB.

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TABLE III. STATISTICAL EVALUATION OF GROUP DATA FOR EACH PREPARATION

PREPARATION	1	2	3	4	5	6	7	8	9A	9B	10	11	THIOMERIN A	THIOMERIN B
Average diuretic response* (DR)	13.1 (10)§	11.6 (5)	17.3 (10)	19.3 (5)	15.2 (5)	10.2 (7)	14.0 (9)	8.2 (9)	15.1 (5)	6.0 (8)	7.2 (10)	5.4 (10)	8.1 (10)	14.7 (10)
Average maximum expected control response† (MCR)	5.5	4.7	5.5	6.3	6.3	4.9	5.5	5.5	4.7	5.7	5.5	5.5	5.5	5.5
Ratio‡ DR/MCR	2.38	2.47	3.15	3.06	2.41	2.08	2.55	1.49	3.21	1.05	1.30	0.98	1.47	2.67

*Average diuretic response: $\frac{\Sigma \text{ Experimental urinary volumes} - \Sigma \text{ control mean urinary volumes.}}{n}$

†$\frac{\Sigma\ 2\ \sigma}{n}$

‡A ratio exceeding 1.0 indicates a probability of chance occurrence of the deviation from control of < 0.05.

§The number of observations with each preparation is indicated in parentheses.

Diuresis Produced by Urine Preparations.—Individual urine volumes, for the five-hour period, following eight preparations of different pooled human urine samples and three preparations of the same sample of lyophilized mare urine will be found in Table II. If an individual urine volume lies outside the range of control mean urine volume for that dog ± 2 standard deviations it is considered to represent a significant effect of the substance injected, with a probability of chance occurrence of less than 0.05.

It will be seen that, with the eight human urine preparations (preparations 1 to 8), only five of sixty injections failed to give a significant increase in urine volume, although there was an increase over the control mean urine volume in each of these five instances.

The results with pregnant mare urine (preparations 9, 10, and 11) were not as consistent as with the human urine preparations. The first trial with preparation 9a showed a significant increase in urine volume in each instance. However, when the experiment was repeated (9b) with this preparation, after seventy-four days' storage in the frozen state, only four of the eight dogs had a significant increase in urine volume over the control mean. With preparation 10, five of ten dogs showed a significant increase, while one dog (No. 25) showed a significant antidiuretic effect of the preparation. With preparation 11, five of ten dogs showed a significant increase in urine volume, and two dogs showed a nonsignificant decrease.

With Thiomerin, given in doses of 1.2 and 2.0 mg. per kilogram of mercury, only three of twenty injections failed to result in a significant increase in urine volume.

In Table III, the combined data for each preparation are evaluated for significance. The *average diuretic response* (DR) for each preparation was calculated by subtracting the sum of the control mean urinary volumes, for those dogs used, from the sum of the experimental urinary volumes; this value then being divided by the number of dogs involved. The average diuretic response is the volume excreted in excess of the average control mean volume. The *maximum expected control response* (MCR) is based on the assumption that each dog used might have excreted, by chance alone, a volume in excess of its control mean volume equal to twice the standard deviation. The maximum expected control response was calculated by dividing the sum of 2σ for those dogs used by the number of dogs involved. If the ratio, DR/MCR, exceeds one, a conservative estimate of the probability of chance occurrence of this deviation would be 0.05. It will be seen, in Table III, that all of the preparations studied, with the exception of preparation 11 and possibly preparation 9b, resulted in a significant increase in the urine volume.

As a further evaluation of the significance of the increases in urine volume following the injection of the various preparations, the grand mean and standard deviation was calculated for eighty control observations, for sixty observations with human urine, and for thirty-three observations with pregnant mare urine. These values were respectively: 15.7 ml. per kilogram ± 3.40, 28.7 ml. per kilogram ± 6.76, and 23.1 ml. per kilogram ± 7.62. Applying

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"Students" t test for the significance of differences between the control mean excretion and the other two mean excretions, the t value for the human urine is 14.931 ($P = <0.0001$), and the t value for pregnant mare urine is 6.895 ($P = <0.0001$). It is concluded that concentrated dialyzed human and pregnant mare urine result in a significant diuresis when injected intravenously in small amounts.

DISCUSSION

Factors Affecting the Excretion of an Oral Fluid Load.—One problem involved in an assay procedure for diuretic substances is the establishment of a relatively normal and reproducible state of hydration. Another problem, which is related to the first, is to present a relatively uniform state of water load, for diuretic substances act best in a state of positive water load.

A number of factors involved in establishing a normal and reproducible state of hydration have been reported.[2] The two main factors were: first, the unreliability of voluntary water consumption in the absence of food in the hours preceding the assay, which was overcome by administering a hydrating dose of water, and the second factor was maintaining the environmental temperature at approximately 22° C. during the hours preceding the assay and during most of the assay procedure.

Two other factors have been tested and reported here. One is the nature of the load presented. It was found that an oral sodium chloride load was excreted much more slowly than an oral water load (68.7 per cent vs. 93.6 per cent recovery in six hours). Also, the variability of recovery, with an oral sodium chloride load, within each animal from day to day in the three time intervals studied was considerable, and it was much greater in the time period of hours 2 through 6 than with a tap water load. Rioch[4] has reported a delay in diuresis in one of four dogs receiving 0.85 per cent NaCl solution in comparison with the diuresis following water ingestion. The second factor observed was that even with the best pre-assay treatment the animals varied considerably in their excretion rates during the first hour. It is probable that remaining variations in hydration state are adjusted during this first hour and are therefore reflected in the urinary volume during this period of time. On the basis of these observations the assay procedure reported here was adopted.

Diuretic Response to Human Urine.—The data reported here clearly show that concentrated dialyzed human urine contains some substance or substances which result in a diuresis, or loss of body water. The average loss of body water with sixty trials on eight preparations was equivalent to 1.3 per cent of the body weight. The potency of the unknown substance must be quite high, since it will be noted that the maximum dry weight injected of these relatively crude preparations was 1.76 mg. per kilogram. There appears to be no direct relationship between the dry weight and the response as can be seen by comparing preparations 5 and 6 (Tables II and III).

The diuresis produced by the human urine compares very favorably with that obtained by the intravenous injection of Thiomerin. It is realized that

this comparison might not be as favorable with an extension of the time of assay because of the known long duration of action of mercurial diuretics.

Diuretic Response to Pregnant Mare Urine.—The diuresis following the injection of pregnant mare urine is not as striking as that following human urine. In fact, in three instances the urinary output was less than the control. However, two factors were noted which might explain the apparent low activity of pregnant mare urine. One fact is that the animals frequently vomited and defecated immediately following the injection of mare urine, indicating some side actions which might affect kidney function indirectly; and the second factor noted was a marked decrease in the rate of urine flow immediately following the injection, which persisted up to approximately one hour. This can be interpreted as indicating the presence of an antidiuretic factor which failed to dialyze out of the preparation.

SUMMARY

Some factors are reported which influence the renal excretion in the dog of an orally administered fluid load, and a procedure for the assay of diuretic substances in this animal is outlined.

Data are presented showing that the intravenous injection of 0.4 ml. per kilogram of dialyzed pooled human urine, which had been concentrated thirty times, results in an average loss of body water of 13.0 ml. per kilogram during a five-hour period. This is a significant loss of body water and is equivalent to 1.3 per cent of the body weight. The maximum dry weight of material injected was 1.76 mg. per kilogram.

Pregnant mare urine, treated in the same manner, produced a much less striking diuresis, which may be due to the presence of an antidiuretic factor which was not dialyzed out completely. However, the diuresis following pregnant mare urine represents a significant loss of body water.

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THE EFFECT OF REPEATED PHLEBOTOMIES IN HEMOCHROMATOSIS

Report of Three Cases

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INTRODUCTION

HEMOCHROMATOSIS is a rare disease of uncertain etiology characterized by widespread deposition of iron-containing pigment throughout the body.[1] Striking concentration usually occurs in the liver and pancreas. The lymph nodes, bone marrow, gastric mucosa, heart, and other organs are less frequently involved. Classically, the condition is characterized by the triad of cirrhosis, diabetes mellitus, and bronzing of the skin; hypogonadism is also a frequent finding. Chemically, there is elevation of the serum iron and saturation of the serum iron-binding protein. The total iron content of the body may be ten to forty times the normal.

The usual course of hemochromatosis is steadily though slowly downhill. Death is generally the result of secondary infection, cirrhosis, diabetes or their complications. Although the discovery of insulin has, to some extent, improved the outlook for these patients, the best available therapy, including the optimum supportive anticirrhotic regimen, has been of little value in altering the progress of the disease.

As a result of the studies of Granick,[2] Finch and associates,[3, 4] and Dubach and co-workers,[5] the normal and abnormal metabolism of iron is now much more clearly understood. There is excellent evidence that the human being has no mechanism of iron excretion except through passive loss by hemorrhage, exfoliation, and the like, with corollary observations to suggest that ordinarily there is an incomplete but definite intestinal barrier[6] to the absorption of iron. That hemochromatosis is a result of a defect in this barrier seems likely; this is in contrast to the earlier idea that the disease is one of abnormal storage, decreased excretion of iron, or a combination of these.

MATERIAL AND METHODS

Since 1946, as a result of routine needle biopsy of the liver in the study of hepatic disease,[7] the diagnosis of hemochromatosis has been established antemortem in four patients at the Ochsner Clinic. Three of these were subjected to repeated phlebotomies in an attempt to mobilize the extensive tissue stores of iron by forcing them to use this tissue iron for the manufacture of hemoglobin. Phlebotomy was contraindicated in the fourth patient because of primary carcinoma of the liver. Bleeding was accomplished by

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removal of 500 ml. aliquots of blood; after separation of the cells from the plasma, the plasma was reinfused and the cells discarded. This procedure was repeated daily until a red blood cell count of 3,000,000 to 3,500,000 per cubic millimeter with a hemoglobin level of approximately 10 to 11 Gm. per 100 ml. of blood was reached. The same procedure was then repeated at intervals of approximately three weeks, or, if circumstances permitted, 500 ml. were removed at intervals of four to eight days in order to maintain this level. An average of 25 to 45 liters of blood was withdrawn annually from each patient. This represented a net annual loss of approximately 12 to 20 Gm. of iron, or about three to five times the total normal body iron content. The only additional therapy was for the cirrhosis and diabetes mellitus; treatment of the former included interdiction of alcohol, a daily diet of 500 Gm. of carbohydrate, 200 Gm. of protein and sufficient fat to render the diet palatable, plus supplementary vitamins in the form of Brewer's yeast (15 Gm.) three times a day, and one Squibb's therapeutic vitamin capsule daily. In only one patient was insulin necessary to control the diabetes.

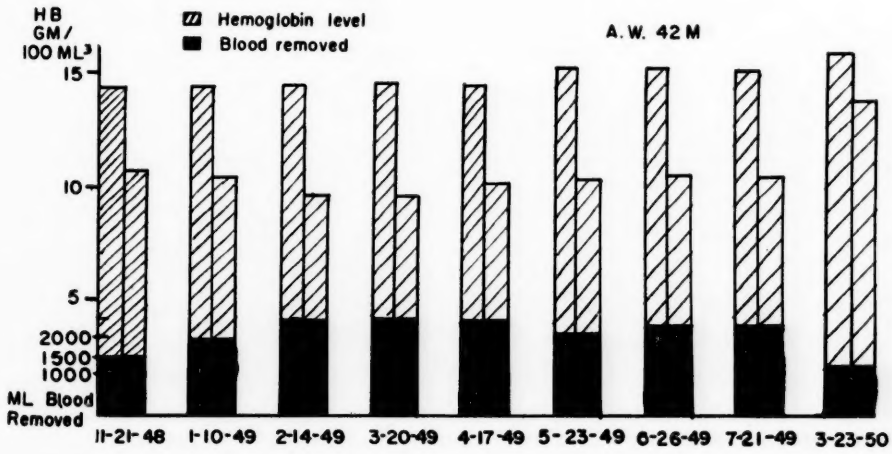


Fig. 1.—Case A. W. Hemoglobin levels before and after each series of phlebotomies. Solid bars represent the total amount of blood removed for each series. Removal of 1,500 ml. produced only one-half as great an effect at the end of the observation period as at the beginning.

RESULTS

A point of great interest in the response of the patients to this procedure was their prodigious ability to manufacture red blood cells and hemoglobin, an observation also noted by Finch and associates,[8] as well as Beyers and coworkers.[9] Without exception the red blood cell counts, hemoglobin levels, and hematocrit values have returned to normal at rates representing the production of 2,000 to 3,000 ml. of whole blood every three weeks for periods as long as two years (Fig. 1). One enthusiastic patient (Case 3) was able to tolerate removal of 18 liters of blood in eighteen weeks and 50 liters of blood in sixty weeks (Fig. 2). Despite this tremendous demand on protein stores, the serum protein levels increased in two of the three patients. The single patient who failed to exhibit increase of the albumin-globulin ratio and the absolute albumin level was an elderly lady who persistently refused to eat her full diet (Case 1).

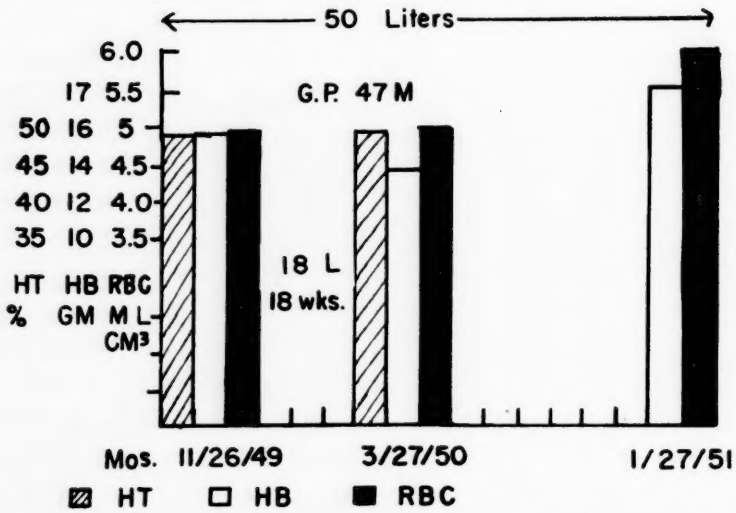


Fig. 2.—Case G. P. Values of red blood cells, hemoglobin, and hematocrit before phlebotomy, after eighteen weeks of treatment, and after sixty weeks. Later in 1951 the hemoglobin concentration was decreased while the red blood count remained unchanged.

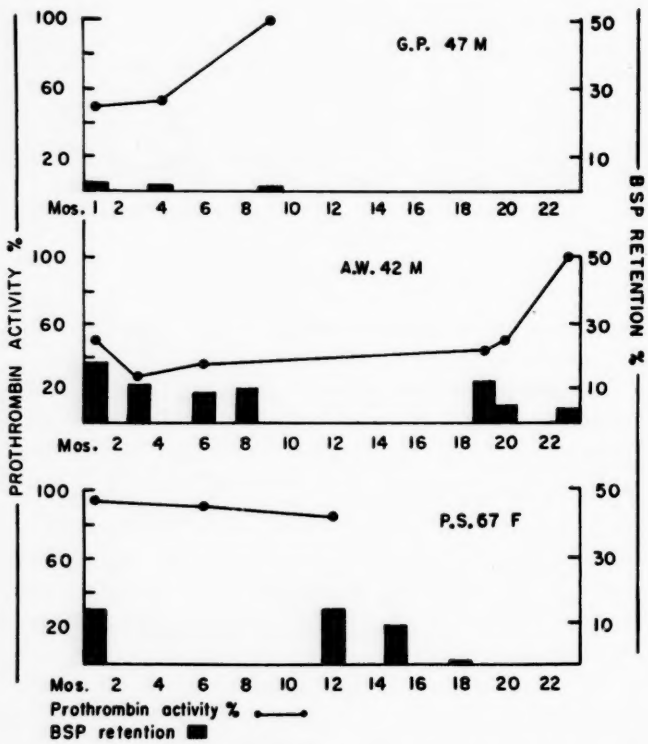


Fig. 3.—Results of liver function studies which were initially abnormal.

Of further interest are the subjective and objective evidences of improvement, manifested by a decrease in size of the liver, increased feeling of well-being, greater ability to work, and general return to useful living. The skin resumed its normal tint, and, in the single patient with electrocardiographic abnormalities, these changes reverted toward the normal.

Liver function returned to normal in the two patients in whom it had been abnormal (Fig. 3), and the two patients with diabetes have shown considerable improvement in ability to utilize carbohydrates, with steady diminution in the fasting blood sugar and in the daily insulin requirement (Fig. 4).

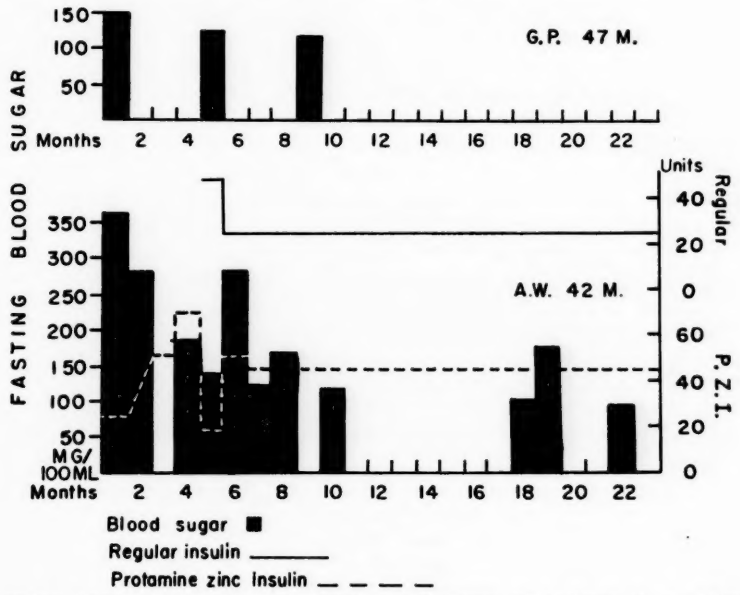


Fig. 4.—Fasting blood sugar levels and insulin requirement of patients G. P. (above) and A. W. (below).

Serial biopsy studies of the liver in all three patients revealed remarkable diminution in the iron content of the liver sections. In the patient treated most recently and most vigorously, the liver section at the present time is essentially free of iron (Fig. 5).

REPORT OF CASES

CASE 1.—Mrs. T. O. S., a white woman aged 69 years, was first seen July 19, 1946, because of recurrent pain in the right upper abdominal quadrant of many years' duration. In 1944 during a cholecystectomy a hard nodular liver was discovered and the gross impression of cirrhosis of the liver obtained, but no biopsy was taken.

On admission to the clinic examination revealed a dusky skin, an enlarged liver which was palpable down to the level of the umbilicus, and a spleen easily palpable 4 cm. below the left costal margin. The sedimentation rate was moderately elevated, and bromsulfalein retention was 10 per cent at thirty minutes following injection of 5 mg. per kilogram of body weight. The total plasma protein level was 6.83 Gm. per 100 ml., with serum albumin of 3.49 Gm. per 100 ml., and serum globulin of 3.34 Gm. per 100 milliliters. Liver biopsy revealed advanced cirrhosis with generalized deposition of iron pigment throughout the section.

Between March 23, 1947, and Feb. 13, 1949, 40 liters of blood were removed and the plasma was reinfused. On Feb. 8, 1949, the red blood cell count was 4,430,000 per cu. mm. and the hemoglobin level 13.9 Gm. per 100 milliliter. On April 28, 1948, there was no bromsulfalein retention at forty-five minutes following injection of 5 mg. per kilogram of body weight. Liver biopsy at that time revealed considerable diminution of iron content, with little change in cirrhosis from the initial specimen obtained on March 23, 1947. The patient had much less abdominal pain, she was much more energetic, and the liver was palpable only about 7 cm. below the right costal margin and no longer tender. The spleen still descended about 3 cm. below the left costal margin.

CASE 2.—A. W., a white man aged 45 years, was first seen on Nov. 28, 1948, because of weakness, abdominal pain, and urinary frequency. He had lost 35 pounds during the previous year. He admitted consuming one and one-half pints of whisky a day for three- to six-day periods over the previous twenty years.

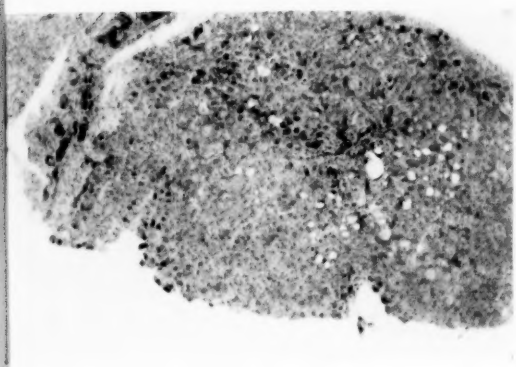
Examination revealed a man with considerable increase in pigmentation of the skin who appeared much older than his stated age. Retinal arteries showed moderate sclerosis. The anteroposterior diameter of the chest was increased, breath sounds were decreased, wheezing was present, and expiratory basal râles were heard bilaterally. A harsh systolic murmur was heard over the apex of the heart. A well-healed thoracotomy scar could be seen in the right posterior lower thorax. The liver was easily palpated 6 cm. below the right costal margin, and the spleen questionably felt 2 cm. below the left costal margin. There was moderate tenderness in the epigastrium and left upper abdominal quadrant. Moderately sized external hemorrhoids and slight edema of the ankles were also noted.

The red blood cell count was 5,160,000 per cu. mm., hemoglobin level 14.4 Gm. per 100 ml., total serum protein 6.23 Gm. per 100 ml., with 2.9 Gm. albumin and 3.3 Gm. globulin, fasting blood sugar 359 and 389 mg. per 100 ml. on two separate determinations, cephalin flocculation reaction 3 plus, bromsulfalein retention 19 per cent at forty-five minutes, and prothrombin level 48 per cent of normal. Cholecystogram showed one calcified stone and a nonfunctioning gallbladder. Liver biopsy on Dec. 4, 1948 (Figs. 5*A* and *C*) revealed severe cirrhosis, moderate fatty infiltration, considerable chronic inflammation, and a tremendous amount of iron pigment in the hepatic parenchymal cells, the connective tissue cells, and the cells of the biliary epithelium.

The patient was given a diet of 200 Gm. protein, 200 Gm. carbohydrate, and 100 Gm. fat. Initially, from 1,500 to 2,500 ml. of blood was removed every three weeks, and then at regular intervals sufficient amounts were removed to maintain the red blood cell count at about 3,500,000 per cubic millimeter.

On April 20, 1949, the red blood cell count was 4,500,000 per cu. mm., with 14.6 Gm. hemoglobin, hematocrit 45 per cent of red blood cells, fasting blood sugar 140 mg. per 100 ml., prothrombin level 50 per cent of normal, total serum protein 6.4 Gm. per 100 ml. with 3.8 Gm. albumin and 2.6 Gm. globulin, bromsulfalein retention 11 per cent at forty-five minutes. Liver biopsy on this date showed considerable diminution in iron pigmentation in all areas, but particularly in the areas of functional parenchymal cells (Fig. 5*B* and *D*). In addition, there was less fatty infiltration, pronounced diminution of chronic inflammation, and complete disappearance of hepatic cellular necrosis. The patient's diet was increased to 500 Gm. carbohydrate and 200 Gm. protein. At this time he was working full time. His insulin requirement had steadily decreased and by Aug. 29, 1950, was down to a mixture of 42 units of regular with 44 units of protamin zinc insulin. Laboratory studies at that time revealed a red blood cell count of 4,020,000 per cu. mm., hemoglobin 12.7 Gm.

Fig. 5.—Photomicrographs of serial liver biopsies in hemochromatosis.
A, Case A. W., before phlebotomy. (Hematoxylin and eosin stain ×100.)
B, Case A. W., after six months of repeated phlebotomies. (Hematoxylin and eosin stain ×100.)
C, Case A. W., before phlebotomy. (Hematoxylin and eosin stain ×400.)
D, Case A. W., after six months. Hematoxylin and eosin stain ×400.)
E, Case G. P., before phlebotomy. (Iron stain ×100.) (Note—Iron stain is potassium ferrocyanide counterstained with fuchsin.)
F, Case G. P., after removal of 50 liters of blood in sixty weeks. (Iron stain ×100.)



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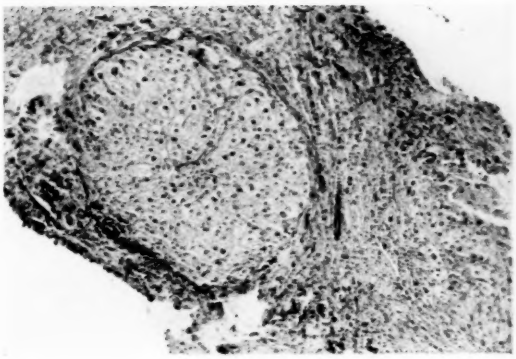

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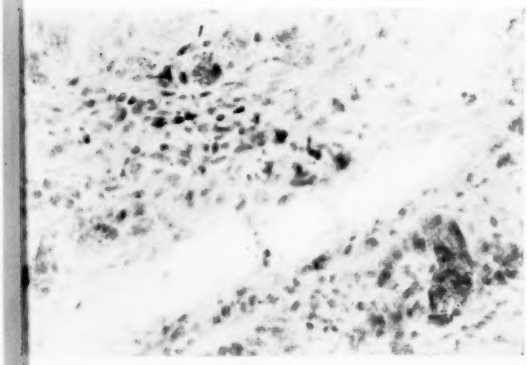

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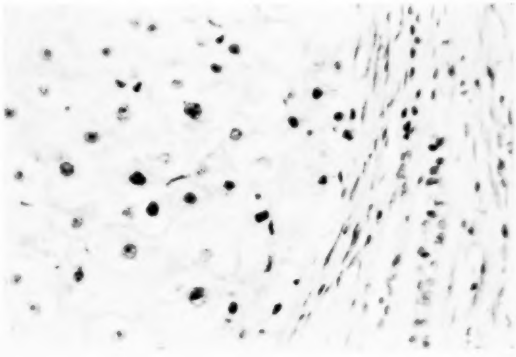

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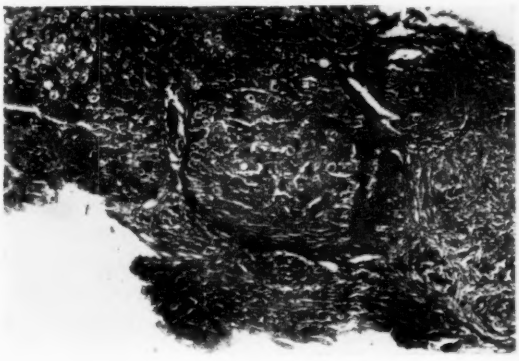

F.

Fig. 5.—*(For legend, see opposite page.)*

per 100 mm., hematocrit 40 per cent red blood cells, total serum protein 6.8 Gm./100 ml., with albumin 4.1 Gm. and globulin 2.7 Gm., bromsulfalein retention 4 per cent at forty-five minutes, and fasting blood sugar 108 mg. per 100 milliliter. Prothrombin level was normal. The liver and spleen could no longer be felt. The patient's complexion was much lighter. He was beginning to find it necessary to add more carbohydrates to prevent episodes of hypoglycemia.

CASE 3.—G. G. P., an osteopathic physician, aged 47 years, was first seen on July 20, 1949, complaining of fatigability and weakness for the previous ten months, and recent incapacity for work. He had consumed increasing amounts of alcoholic beverages for the previous five years and had had episodes of confusion and disorientation five months before admission.

Initial examination revealed a blood pressure of 144/100 in both arms. The entire skin had a bluish cast, and discoloration of the skin around the ankles was rather pronounced. The liver could be felt about 8 cm. below the right costal margin; the spleen was not palpable.

Laboratory studies revealed a red blood cell count of 4,900,000 per cu. mm., hemoglobin 14.9 Gm. per 100 ml., hematocrit 41.5 per cent of the red blood cells, fasting blood sugar 150 mg. per 100 ml. (average of several determinations), prothrombin level 49.6 per cent, serum proteins total 5.8 Gm. per 100 ml., albumin 3.2 Gm., globulin 2.6 Gm., bromsulfalein retention 4 per cent at forty-five minutes. Electrocardiogram revealed low T waves in leads 1 and 2 and V4 through V6. Liver biopsy revealed hemochromatosis with extensive deposition of iron pigment throughout the sections and moderately advanced cirrhosis (Fig. 5*E*).

An anticirrhotic supportive regimen was instituted and phlebotomies were begun; 33 liters of blood were removed during the ensuing thirty-five weeks. The patient reported a tremendous increase in energy and resumed working full time.

When next seen on March 27, 1950, the bromsulfalein retention was 3 per cent at forty-five minutes, total serum protein 6.7 Gm. per 100 ml. with 4.0 Gm. of albumin, 2.7 Gm. of globulin, prothrombin level 100 per cent, fasting blood sugar 123 mg. per 100 ml., hemoglobin 13.7 Gm. with a red blood cell count of 5,000,000 per cubic millimeter. Liver biopsy at this time revealed a tremendous decrease in the concentration of iron pigment in all areas but again most pronounced in the areas of healthy parenchyma with complete disappearance of inflammatory reaction within the hepatic lobules.

When again seen, March 5, 1951, the patient's complexion was normal, the liver could barely be felt 2 cm. below the costal margin on deep inspiration, and he was working full time and felt perfectly well. Total serum protein was 5.5 Gm. per 100 ml., with 4.0 Gm. of albumin, 1.5 Gm. of globulin; bromsulfalein retention was 4 per cent at forty-five minutes, fasting blood sugar 116 mg., hemoglobin 10.4 Gm. per 100 ml., and red blood cell count 3,850,000 per cubic millimeter. Fifty liters of blood had been removed since July 29, 1949. Electrocardiogram showed much improvement and was essentially within normal limits. Liver biopsy revealed a picture of inactive cirrhosis with essentially no iron pigmentation (Fig. 5*F*).

DISCUSSION

The etiology of hemochromatosis is still far from clear. The interesting demonstration of its frequency in South Africa in association with grossly deficient diets[10, 11] (largely corn grits) now is explained by the experimental studies of Kinney and associates,[12, 13] who demonstrated that rats on this type of diet absorb increased amounts of iron; this is apparently due to the low phosphorus content of the diet. Full-fledged experimental hemochromatosis has been produced by this regimen, together with the addition of iron in the diet. This hypothesis has been given additional confirmation by the demonstration of high iron content in the diet of the Bantu in South Africa.[14] The

question of whether iron alone is a damaging agent in these quantities in the tissues is of considerable interest. Evaluation of the clinical picture and pathologic state of full-blown hemochromatosis and of hemosiderosis resulting from repeated blood transfusions[15] is at least suggestive of this possibility. However, its experimental counterpart is as yet lacking. The remarkable clinical improvement which our patients have shown as a result of simple iron mobilization also suggests that these extensive iron deposits are in themselves capable of inducing symptoms and accelerating the progress of fibrotic changes in the parenchymal organs. Whether repeated bleeding does more than reduce the hemochromatosis to ordinary hepatic cirrhosis must await further observation. Nonetheless, it has been conclusively shown that in hemochromatosis the tissue iron is readily available for hemoglobin synthesis. The implementation of routine study of hepatic problems by needle biopsy has unquestionably stimulated our interest in this condition and has directly provided the clinical material which has made these observations possible, by establishing the diagnosis of hemochromatosis ante mortem in these patients.

SUMMARY

Three patients with hemochromatosis treated by repeated phlebotomies have exhibited ability to mobilize the excess tissue iron, tremendous capacity to regenerate red blood cells and hemoglobin, pronounced subjective improvement, diminution in size of the liver, improvement in liver function and in carbohydrate metabolism, and considerable decrease in the concentration of iron in the liver as shown by repeated biopsy sections of the liver.

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THE FLOCCULATION TESTS; A CONTRIBUTION TO THE STUDY OF THEIR MECHANISM

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THE introduction of the so-called "Flocculation Tests" in the diagnosis of jaundice has been an important contribution. These include the cephalin flocculation test, thymol turbidity and flocculation, colloidal gold and colloidal red tests, and others. The simplicity of the techniques and the close correlation of results with the clinical diagnosis have established their superiority to many other tests, and have also stimulated the investigation of factors involved in their production.

Since the value of these tests in clinical work has been proved, their positivity has been attributed to changes, both quantitative and qualitative, in the serum proteins. During the last few years electrophoretic analysis of serum proteins has partly confirmed this hypothesis.

Human blood serum contains substances which determine the occurrence of flocculation or turbidity in these tests, and other substances which inhibit these phenomena. Normally, the equilibrium of these opposing factors results in a negative test, whereas in certain pathologic conditions the balance is altered and positive tests occur.

Gray[1] was the first investigator to employ pure protein fractions, separated by electrophoresis, and to demonstrate the flocculating effect of gamma-globulin and the inhibiting effect of albumin in the colloidal gold test. Kabat and associates[2] confirmed these results and extended them to the cephalin-cholesterol test. Moore and his co-workers[3] made an important contribution when they established that the inhibitory effect upon flocculation of albumin from patients suffering from diffuse, parenchymatous liver disease was less marked than that of the albumin coming from normal individuals. The importance of the "quality" of the albumin was thus demonstrated. These investigators also found that albumin itself, of whatever nature, lacks flocculating power, and that gamma-globulin always possesses this power whether or not it is derived from an individual suffering from liver disease. Therefore, as far as gamma-globulin is concerned, "qualitative" differences would be nonexistent or nonoperative.

In another paper the same authors[4] demonstrate that the precipitating effect of gamma-globulin occurs in the following circumstances: (a) when its quantity is such that the normal quantity of albumin is insufficient to inhibit flocculation, (b) when the quantity of albumin is sufficiently below the (normal) level at which inhibition occurs, and (c) when the quality of the albumin is deficient and its neutralizing power thus impaired.

In 1944 MacLagan[5] introduced a new test, that of the thymol turbidity. Recant and associates,[6] after extracting the serum lipids by freezing in the presence of ether, found that the thymol test became negative, whereas Hanger's test remained unaltered. Gamma-globulin isolated from either normal or pathologic sera, contrary to what is observed in other floccula-

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tion tests such as Hanger's and collodial gold, did not give positive thymol reactions. Normal albumin inhibited the reaction, a fact not observed when albumin from patients with hepatitis was employed.

Cohen and Thompson[7] used electrophoresis to analyze sera (obtained from normal individuals and from hepatitis patients) before and after treating them with the thymol reagent. The thymol reaction produces precipitation, leaving a supernatant fluid when the test is positive. Once this precipitate is removed, a decrease of beta-globulin is observed in the supernatant fluid, suggesting that thymol turbidity is conditioned by the presence of beta globulin in the serum.

In a more recent paper Hanger[8] states that the determining factor of positive cephalin-cholesterol tests in hepatic disease lies in the alteration of the albumin fraction, not in the albumin itself but in another component moving with this fraction. Normally albumin acts as an inhibitor and, if it were absent, all sera would display positive reactions.

The same paper states that positive thymol tests depend upon changes provoked by gamma-globulin in the presence of lipids. Thus gamma-globulin isolated from hepatitis serum gives a negative thymol reaction which becomes positive if lipids or normal whole serum is added.

Kunkel and Hoagland[9] stressed the importance of lipids in the thymol turbidity test, but considered that the essential factor in determining positive reactions is the presence of gamma-globulin; the lipids merely influencing the intensity of the test.

MacLagan and Bunn,[10] by means of an original electrophorectic method using very little serum, have isolated protein fractions from sera of two normal individuals and two cases of infectious hepatitis. Evaluating the effect of these fractions on the reagents used in several flocculation tests, they conclude: (a) normal or pathologic gamma-globulin acts as a flocculating agent in Hanger's test, in the colloidal gold, Takata-Ara and Thymol-cephalin turbidity tests. (b) Hepatitis gamma-globulin is even more effective in all these reactions. Moreover, thymol-cephalin precipitation only occurs with this type of gamma-globulin, and not with that obtained from normal individuals. (c) Normal albumin inhibits the colloidal gold, cephalin-cholesterol and thymol-cephalin reactions in contrast to albumin from hepatitis, which does not inhibit the thymol-cephalin tests. (d) Alpha-plus beta-globulin fractions inhibit the colloidal gold reaction, but, when coming from hepatitis serum, they render the cephalin cholesterol reaction positive. As regards the thymol reaction, the authors link its positivity with the presence of gamma-globulin, and state that when this is derived from hepatitis serum, the reaction is always positive; and when it comes from normal serum there is no reaction. Identical results occur when the thymol-cephalin reagent is used.

Bauer[11] studied the flocculation tests with fractions isolated by electrophoresis, and concluded that not all of the conditioning factors are known. He found that no individual fraction gave a positive thymol reaction. Positivity occurred only when either mixtures of lipoproteins or gamma-globulin were employed.

Ducci[12] noted that by employing fractions isolated by chemical methods, it could be demonstrated that normal gamma-globulin produces less flocculation when mixed with serum from obstructive jaundice than when it is mixed with normal serum; from this he concluded that serum from obstructive jaundice has a greater inhibitory effect upon flocculation than has normal serum.

Cabello[13-14] discussed the distilled-water dilution test, which he described simultaneously with Dreyfuss[15] and associated its mechanism with the precipitation of the serum euglobulins. Euglobulins, as determined by electrophoretic analysis, corresponded with the beta- and gamma-globulin fractions.

From all of these studies the following conclusions emerge: (a) Albumin is a "preventive" factor in flocculation. (b) This effect upon flocculation is conditioned by the quality of the albumin, because greater "protection" is observed when the albumin comes from a normal individual and not from patients with diffuse parenchymatous disease of the liver. (c) Gamma-globulin is a definite flocculating agent. (d) Opinions are divided as to the influence of the "quality" of gamma-globulin: Some investigators[3] considering that qualitative changes are important, while others[10] deny this.

In the presence of these contradictions and given the possibility of isolating sufficient quantities of the protein fractions by a method already described,[16] we decided to undertake the present investigations.

Flocculation tests, (a) cephalin-cholesterol, (b) colloidal gold, (c) colloidal red, (d) thymol (turbidity and precipitation) and dilution or double-distilled water (turbidity and precipitation) are performed as a routine in our clinic for the differential diagnosis of jaundice. These tests provide the basis for the present studies.

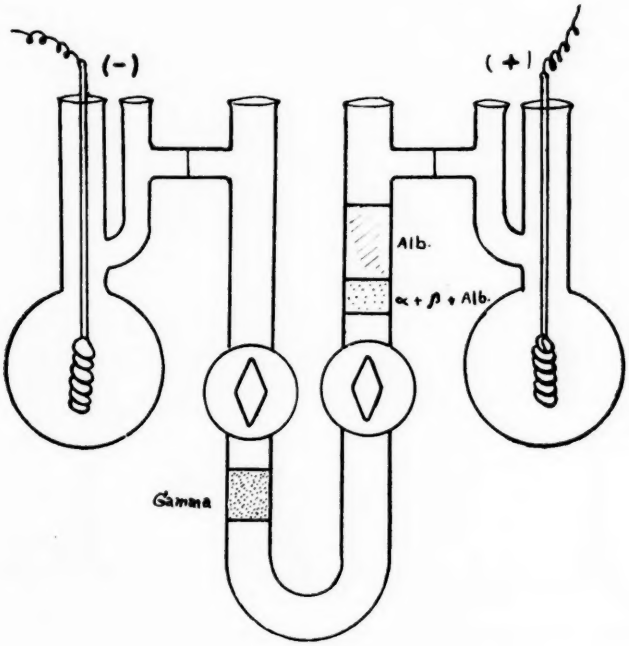

Fig. 1.

MATERIALS AND METHOD OF STUDY

Separation of the Protein Fractions of Sera by Electrophoresis.—This was achieved by means of a method previously described,[16] the apparatus for which is shown in Fig. 1. It is based on the principle of the Tiselius apparatus, and consists of a U-tube, in which the material for fractionation is placed; an electric current passes through and the albumin, with its greater mobility, moves to the right (or ascending) arm of the U-tube, while the much slower gamma globulin accumulates in the lower part of the left (or descending) arm of the tube. The subsequent extraction of these pure fractions is easily effected by pipettes or needles.

Material Employed.—Electrophoresis was performed in two groups of sera, as follows: (a) specimens obtained from twenty-five apparently healthy individuals between the ages of 24 and 35 years; (b) a pathologic group including: (1) sera from three cases of infectious hepatitis with marked icterus, (2) sera from two cases of cirrhosis of the liver, (3) sera from three cases of posthepatic or obstructive jaundice (two due to lithiasis of the common duct, and one to cancer of the biliary ducts).

Hepatitis sera were obtained during the height of the disease; those that gave intensely positive flocculating tests with whole serum were then selected (an identical method was applied to cirrhotic sera). The samples provided by patients with obstructive jaundice gave negative flocculation tests.

Blood samples of 200 ml. each were obtained, although the removal of this quantity was objected to by the majority of the patients. Objections were overcome by giving each patient (especially cirrhotics) a transfusion of whole blood immediately after each venesection. From each sample about 50 ml. of serum was obtained, part of which was subjected to fractionation, part to the chemical analysis of the proteins and flocculation tests, and a small amount was reserved for determining the electrophoretic pattern.

Degree of Purity and Quantity of Isolated Fractions.—Using the above-described method, albumin- and gamma-globulin fractions were isolated in what might be termed as a 100 per cent degree of purity, which in each case was controlled by electrophoresis (Fig. 2).

By charging the U-tube with 33 ml. of serum, from 4 to 8 ml. of albumin solution and from 2 to 6 ml. of gamma-globulin solution were isolated after electrophoresis. Average absolute concentrations were as follows: for albumin 2.86 ± 0.44 Gm. per cent and for gamma-globulin 2.56 ± 0.53 Gm. per cent.

Solutions were obtained in phosphate buffer with a pH of 7.4. Up to the present time the separation of the lipoglobulins (alpha- and beta-globulin) has proved unsatisfactory.

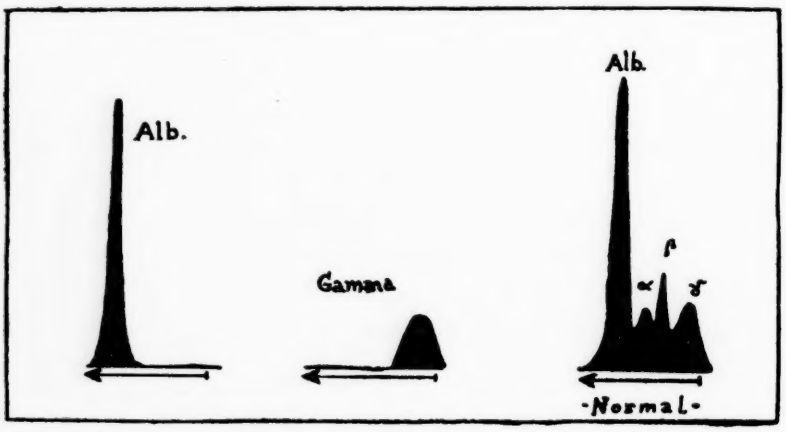

Fig. 2.

Use of the Electrophoretically Isolated Fractions in the Performance of the Flocculating Reaction.—Fractions of confirmed purity, isolated by electrophoresis, were used in the following tests:

Hanger's cephalin-cholesterol test,[17] MacLagan's colloidal gold,[18] Ducci's colloidal red reactions,[12] MacLagan's thymol turbidity,[5] and Cabello's[14] twice-distilled water tests. The first experiments were performed with a 4 per cent solution of albumin (with phosphate buffer and a 7.4 pH) and a 1.5 per cent solution of gamma-globulin in the same medium. Each test was done repeatedly, both with fractions isolated from normal sera and fractions from the sera of patients suffering from hepatitis, from cirrhosis of the liver, and from obstructive jaundice.

No positive results were obtained with albumin, even when large quantities of 4 per cent albumin solutions of either normal or pathologic albumins were employed. Results obtained with gamma-globulin will be described later.

Hanger's Cephalin-Cholesterol Reaction.—This is the test most sensitive to gamma-globulin. Even quantities as small as 0.001 ml. of the 1.5 per cent solution provoke some flocculation of the suspension. Hepatitis and cirrhosis gamma-globulins appear to have a greater flocculating potency than normal or obstructive jaundice gamma-globulin (Table I).

MacLagan's Colloidal Gold and Ducci's Colloidal Red Reactions.—These reactions gave parallel results, and so were examined together. As in the previous reaction, the flocculating power of gamma-globulin—either normal or pathologic—was again apparent.

TABLE I. CEPHALIN-CHOLESTEROL REACTION

1.5% SOLUTION GAMMA-GLOBULIN	NORMAL	OBSTRUCTIVE JAUNDICE	HEPATITIS	CIRRHOSIS
0.001 ml.	+	(−)	+ +	+ +
0.005 ml.	+ + +	+ + +	+ + +	+ + +

Hepatitis gamma globulin in 1.5 per cent gave flocculating effects from quantities ranging from a minimum of 0.004 ml. to a maximum of 0.04 ml., whereas the range displayed by cirrhotic gamma-globulin lay between 0.01 to 0.05 ml., and that of obstructive jaundice gamma-globulin between 0.08 and 0.25 ml. (a range very similar to that of normal gamma-globulin) (Table II).

TABLE II. COLLOIDAL GOLD AND COLLOIDAL RED REACTIONS

1.5% SOLUTION GAMMA-GLOBULIN (ML.)	NORMAL	OBSTRUCTIVE JAUNDICE	HEPATITIS	CIRRHOSIS
0.003	0	0	0	0
0.004	0	0	1	0
0.005	0	0	2-3	0
0.01	0	0	3-4	1
0.02	0	0	3-4	3
0.03	0	0	4	3
0.04	0	0	5	4
0.05	0	0	5	5
0.06	0	0	5	5
0.07	1	0	5	5
0.08	1	1		
0.09	2	2		
0.1	2	2-3		
0.15	4	4		
0.2	4	4		
2.25	5	5		

It therefore appeared that in these reactions gamma-globulin from patients with parenchymatous disease of the liver was about twenty times more potent than normal (or obstructive jaundice) gamma-globulin.

MacLagan's Thymol Reaction.—Both turbidity (T) and precipitation (P) were examined. Pure gamma-globulin was employed exactly as in the previous tests. Results are summarized in Table III.

TABLE III. THYMOL REACTION

1.5% SOLUTION GAMMA-GLOBULIN	NORMAL		OBSTRUCTIVE JAUNDICE		HEPATITIS		CIRRHOSIS	
	P.	T.	(P)*	(T)†	P	T.	P.	T.
(ML.)								
0.03	(−)	0	(−)	0	−	0	−	0
0.04	(−)	0	(−)	0	−	0	−	0
0.05	(−)	0	(−)	0	+	2.8	+	2.4
0.06	(−)	0	(−)	0	++	3.4	+	2.4
0.07	(−)	0	(−)	0	+++	6.4	++	2.6
0.08	(−)	0	(−)	0	+++	4.4	++	3.0
0.09	(−)	0	(−)	1.4	+++	6.4	++	4.2
0.1	(−)	0	(−)	1.4	+++	6.8	++	4.2
0.15	(−)	0	(−)	1.4	+++	7.3	+++	6.4
0.2	(−)	1.4	(+)	3.6	+++	8.0	+++	7.0
0.25	(−)	1.8						
0.3	(−)	3.0						
0.4	(−)	4.0						
0.5	(+)	5.0						

*Precipitation.
†Turbidity.

It must be emphasized that the reaction is positive when hepatitis—or cirrhosis—gamma-globulin is used, but remains persistently negative when this is substituted by normal or obstructive globulin even in large quantities, thus confirming MacLagan's finding.[10]

Dilution, or Twice-Distilled Water Reaction.—This simple test was described simultaneously by Cabello and associates[13, 14] and Dreyfuss.[15] Gamma-globulin, whether isolated from normal or pathologic sera, gave a positive reaction. Nevertheless, flocculation was greater when gamma-globulin came from cases of cirrhosis or hepatitis. Obstructive jaundice-globulin provoked less flocculation than the former but more than that of normal globulin—which had this effect only when large quantities were used. As in the previous test, readings both of turbidity and precipitation in twenty-four hours were made (Table IV).

TABLE IV. DILUTION, OR TWICE-DISTILLED WATER TEST

1.5% SOLUTION GAMMA-GLOBULIN (ML.)	NORMAL		OBSTRUCTIVE JAUNDICE		HEPATITIS		CIRRHOSIS	
	P.*	T.†	P.	T.	P.	T.	P.	T.
0.01	–	0	–	0	–	0	–	0
0.02	–	0	–	0	–	0	–	0
0.03	–	0	–	0	+	0.9	+	0.5
0.04	–	0	+	0.9	++	2.6	++	1.9
0.05	+	1.0	++	2.0	++	2.6	++	2.8
0.06	+	1.0	++	2.0	++	2.6	++	3.8
0.07	+	1.5	++	2.0	+++	3.8	+++	3.8
0.08	+	1.5	++	2.8	+++	4.0	+++	3.8
0.09	+	2.2	++	2.8	+++	4.1	+++	3.8
0.1	+	2.6	++	2.8	+++	4.1	+++	4.0
0.15	+	2.8	++	3.4				
0.2	+	2.8	+++	3.4				
0.25	+	2.8	+++	3.6				
0.3	+	5.3						
0.4	++	7.4						
0.5	+++	8.7						

*Precipitation.
†Turbidity.

Summarizing our investigations with electrophoretically pure albumin and gamma globulin: (1) albumin showed no flocculating activity; (2) gamma-globulin showed flocculating activity, enhanced when its source was a case of parenchymatous liver disease.

OTHER OBSERVATIONS

Further studies were made of the flocculating activity of gamma-globulin. First, normal serum was added to gamma-globulin from cases of (a) hepatitis, (b) obstructive jaundice, and (c) cirrhosis. Results are expressed in Table V.

TABLE V. FLOCCULATING ACTION OF NORMAL SERUM COMBINED WITH GAMMA-GLOBULIN FROM: (A) HEPATITIS, (B) OBSTRUCTIVE JAUNDICE, (C) CIRRHOSIS

	GOLD	RED	HANGER	THYMOL		WATER OR DILUTION	
				P.*	T.†	P.*	T.†
0.5 ml. normal serum plus 0.3 ml. gamma hepatitis	2	2	(++)	(–)	8.2	(+)	5
0.5 ml. normal serum plus 0.3 ml. gamma obstructive jaundice	0	0	(–)	(–)	4.7	(–)	3
0.5 ml. normal serum plus 0.3 ml. gamma cirrhosis	3	3	(++)	(–)	6.6	(–)	4.9

*Precipitation.
†Turbidity in MacLagan units.

TABLE VI. ACTION OF 4 PER CENT SOLUTION OF NORMAL ALBUMIN UPON 1.5 PER CENT SOLUTIONS OF: (A) NORMAL, (B) HEPATITIS, (C) OBSTRUCTIVE JAUNDICE GAMMA-GLOBULIN

	GOLD	RED	HANGER	THYMOL		WATER OR DILUTION	
				P.*	T.†	P.*	T.†
0.5 ml. hepatitis gamma-globulin plus 0.5 ml. normal albumin	3	5	(+++)	(+++)	7.2 units	(+++)	3 units
0.5 ml. obstructive jaundice gamma globulin plus 0.5 ml. normal albumin	0	0	(−)	(−)	1.3	(−)	1
0.5 ml. normal gamma globulin plus 0.5 ml. normal albumin	0	0	−	(−)	2.0	(−)	1

*Precipitation.
†Turbidity.

It, therefore, appeared that gamma-globulin from cases of diffuse parenchymatous disease of the liver had a flocculating potency not inhibited by whole normal serum in quantities that were inhibitory when normal gamma-globulin was used.

This experiment also revealed an interesting fact: precipitation with thymol and water were not, as expected, positive with hepatitis and cirrhosis globulin. Thus, normal serum appeared to inhibit precipitation, but not turbidity.

Further proof of the superiority of hepatitis globulin over obstructive jaundice-globulin in the production of flocculation was supplied by the following observations:

Hepatitis globulin proved more resistant to the inhibiting reaction of a 4 per cent albumin solution. Table VI summarizes these observations.

These experiments with pure albumin appeared satisfactory in so far as they demonstrated the qualitative importance of gamma-globulin in the production of flocculation. They must, however, be considered as preliminary, as difficulties were encountered regarding the dilution of the material. These difficulties have been overcome in more recent work.

TABLE VII. ACTION OF NORMAL GAMMA GLOBULIN WHEN ADDED TO: (A) WHOLE NORMAL SERUM, AND (B) OBSTRUCTIVE JAUNDICE SERUM

1.5 % SOLUTION GAMMA-GLOBULIN (ML.)	GOLD AND RED	HANGER	THYMOL		WATER OR DILUTION	
			P.*	T.(μ)†	P.*	T.(μ)†
(a) 2.8 Per Cent Solution Normal Gamma Globulin + 0.5 Ml. Normal Serum						
0.05	0	−	−	2.8	−	1.1
0.1	0	−	−	2.8	−	1.1
0.15	0	−	−	4.5	−	1.1
0.2	0	+	−	4.5	+	1.7
0.25	1	++	−	6.0	++	5.7
0.3	5	+++	−	6.0	+++	6.7
0.35	5	+++	−	6.0	+++	7.7
0.4	5	+++				
0.45	5	+++				
(b) 2.8 Per cent Solution Normal Gamma-Globulin + 0.5 Ml. Whole, Obstructive Jaundice Serum						
0.05	0	−	−	2.8	++	9
0.1	0	−	−	2.8	++	9
0.15	0	−	−	2.8	+++	10
0.2	0	−	−	4.0	+++	10
0.25	0	−	−	4.7		
0.3	0	+	−	4.7		
0.35	0	+	−	5.5		
0.4	1	++				
0.45	3	+++				

*Precipitation.
†Turbidity.

Finally, using gamma-globulin from normal subjects, it was possible to confirm Ducci's[12] finding, i.e., that whole serum from obstructive jaundice contains some component (probably nonprotein according to Ducci) that hinders its flocculation when an excess quantity of normal gamma-globulin is added. In these experiments we used a 2.8 per cent solution of normal gamma-globulin and found this increased "protective" action of obstructive jaundice serum in the colloidal gold, colloidal red, cephalin-cholesterol, and thymol reactions. Regarding the twice-distilled water reaction (not investigated by Ducci) the observed effect was paradoxical: obstructive jaundice serum added to normal gamma-globulin produces more intense flocculations than did normal serum (Table VII).

DISCUSSION

By using pure protein fractions in the performance of the various flocculation tests (currently employed for the differential diagnosis of jaundice) we established certain facts that appear to be fundamental in the comprehension of the mechanism of these tests; i.e., the importance of the quality of gamma-globulin.

Electrophoretically pure albumin (whether normal or pathologic) shows no flocculating action. Gamma-globulin from normal sera flocculates cephalin-cholesterol suspension produces positive colloidal gold—and red—reactions and turbidity and precipitation when added to distilled water. At the concentrations used, it does not, however, determine turbidity or precipitation with thymol.

Gamma-globulin from pathologic sera (diffuse parenchymatous diseases of the liver) displays a greater flocculating potency in all the tests. Gamma-globulin from cases of obstructive jaundice behaves, in general, like normal gamma-globulin.

From these findings and from the available published facts, we conclude that, although the mechanism of flocculation is highly complex, certain decisive factors involved are now known.

In normal sera, negative reactions are conditioned by an equilibrium between the flocculation and turbidity factors (represented mainly by gamma-globulin with a weak flocculating potency) and the strongly inhibitory factors (represented by albumin or other substances with similar electrophoretic behavior).

In infectious hepatitis and hepatic-cirrhosis sera, conditions obtain which favor the positivity of the tests. In the first place, as we believe to have demonstrated, the quality of gamma-globulin is altered, acquiring a much greater flocculating potency than normal gamma-globulin. If this change of quality (which we consider most important) is associated with an impaired quality of the inhibitory factors (albumin or other substance),[3, 4] optimal conditions for positive reactions arise. Moreover, when pathologic sera are involved, quantitative changes reinforce the qualitative ones. Our electrophoretic studies had already revealed the increase in quantity of gamma-globulin, coupled with a significant decrease of albumin in hepatitis—and cirrhosis—sera[20, 21] (Table VIII).

Finally, in obstructive jaundice, the normal or diminished flocculating gamma-globulin tends to produce negative reactions, although its quantity

TABLE VIII. MEAN PROTEIN CONCENTRATION IN: (A) NORMAL SERA,[19] (B) HEPATITIS SERA,[20] (C) LAENNEC'S CIRRHOSIS SERA[21] ELECTROPHORETIC FRACTIONATION

	NORMAL (PER CENT)	HEPATITIS (PER CENT)	CIRRHOSIS (PER CENT)
Albumin	57.6 ± 0.608	45.0 ± 1.28	30.5 ± 3.02
α-globulin	7.4 ± 0.405	5.5 ± 0.46	5.8 ± 2.5
β-globulin	14.3 ± 0.44	18.9 ± 0.77	14.0 ± 4.1
γ-globulin	20.7 ± 0.639	30.0 ± 1.43	49.4 ± 5.6

may be normal or increased. The quality of albumin in these cases is yet unknown, but its quantity is often reduced. To these factors, in biliary obstruction, another factor (as yet unknown) lending increased resistance to the flocculating activity of gamma-globulin must be added. We refer to Ducci's[12] finding, which we have confirmed in the majority of the tests.

We must emphasize, before closing, the similarity of many of our conclusions to those of MacLagan[10] and remark that we were unacquainted with his paper when commencing most of these investigations. Results are, therefore, doubly confirmed and amplified.

SUMMARY

By a simple electrophoretic method, pure albumin and gamma-globulin fractions (in sufficient quantity for experimental purposes) have been separated from normal human sera and from sera of cases of infectious hepatitis, cirrhosis of the liver, and obstructive jaundice.

Degree of purity of these fractions is confirmed by other electrophoretic patterns.

The following tests: cephalin-cholesterol flocculation, colloidal gold, colloidal red, thymol and twice-distilled water, are performed using these pure fractions.

The lack of flocculating activity of albumin, whether normal or pathologic, is confirmed.

The flocculating potency of normal gamma-globulin is confirmed in the cephalin-cholesterol, colloidal gold, and red test, and the distilled water test, but is absent (with the concentration used here) in the thymol test.

Gamma-globulin from hepatitis and cirrhosis sera has a higher flocculating potency than normal gamma-globulin in all the tests including thymol.

Gamma-globulin from obstructive jaundice sera (as is the case with normal sera) determines positivity of all the tests except thymol.

The fact that serum from obstructive jaundice has a greater antiflocculating potency than normal serum is confirmed.

The known factors which favor or impede flocculation are reviewed and discussed.

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SERUM CHOLINESTERASE ACTIVITY IN LIVER DISEASE

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SERUM cholinesterase activity has been reported to be reduced in parenchymal liver disease.[1, 2] Vorhaus and associates[3, 4] have proposed the method of Michel[5, 6] to help differentiate obstructive from hepatocellular jaundice, and state that when performed serially the test is an aid to prognosis in cirrhosis and acute hepatitis.

Our experience with this test in 115 cases of hepatobiliary disease and 168 control subjects agrees partially with these conclusions, but differs in certain important aspects.

MATERIAL AND METHODS

This investigation was conducted in an Army Hospital. The patients and controls were selected for study as outlined in Table I. Serial observations were made whenever possible. Except where indicated, only the first test values obtained in each patient were included in this report.

Cholinesterase was performed by the technique of Michel.[5] The following liver function tests were also performed; serum bilirubin,[6] thymol turbidity,[7] cephalin flocculation,[8*] zinc sulfate turbidity,[9] gamma-globulin turbidity,[10] and bromsulfalein retention.[11]

RESULTS

Normal Subjects and Hospital Patients Without Evident Liver Disease.—A statistically significant decrease in cholinesterase activity from the normal was observed in the group of surgical cases ($t = 3.65$)† and in the battle casualties ($t = 4.31$).† As indicated in Fig. 1, however, individual values in all groups overlapped considerably, and a wide range of values was encountered. Values above 0.7 Δ pH units per hour were considered to be in the normal range.

Acute Viral Hepatitis.—As previously reported, serum cholinesterase levels tended to be depressed during the first week of acute hepatitis and increased in a characteristic way as the patient recovered (Figs. 1 and 2). Regardless of the initial values, this increase occurred in twenty-nine of the thirty patients included in the series (Table II).

Our experience with thirty-six additional patients with acute hepatitis, who were not included in this series because of incomplete records, was substantially the same as above.

Two of the total number suffered a definite clinical relapse while under observation (Figs. 3 and 4). In one case the serum cholinesterase drop cor-

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*Read at twenty-four hours.
†Student's t Test.

responded to the time of the clinical relapse (Fig. 3). In the other case the relationship was equivocal (Fig. 4).

Chronic Viral Hepatitis.—In twenty cases of proved chronic hepatitis the serum cholinesterase levels did not differ significantly from those in normals, ($t = 2.16$) (Fig. 1).

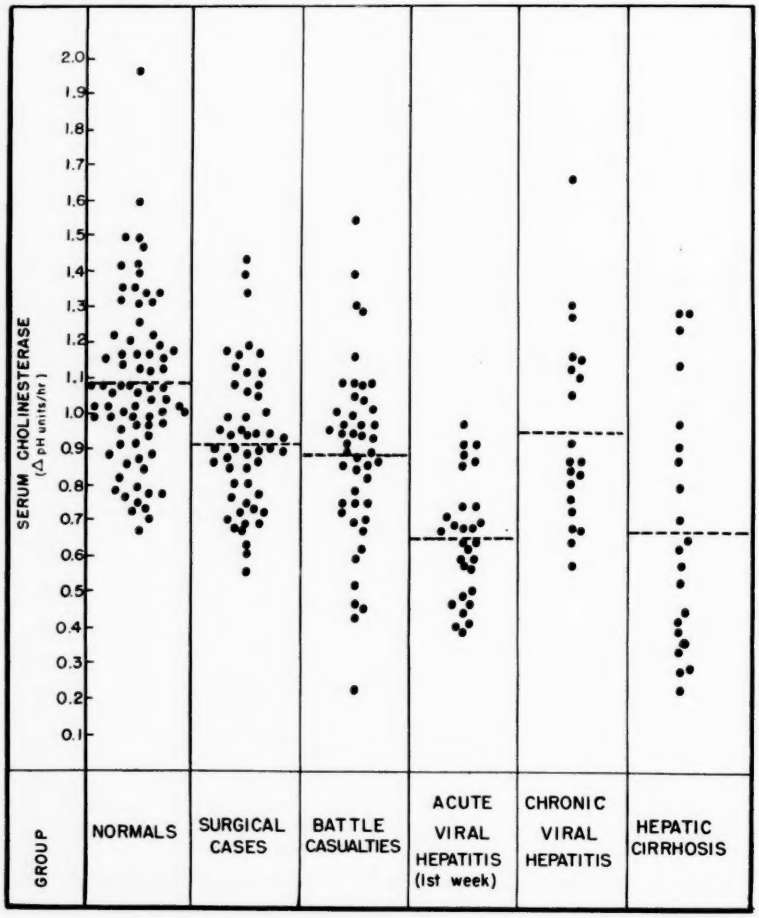

Fig. 1.—Scattergram depicting serum cholinesterase values in control subjects and patients with hepatobiliary disease.

Obstructive Jaundice.—In four of six cases of obstructive jaundice, the serum cholinesterase was abnormally low. Five patients had obstruction of the biliary passages due to malignancies, while one had a common duct calculus. These are considered to be too few cases from which to draw conclusions.

Hepatic Cirrhosis.—In cirrhosis of the liver the serum cholinesterase values were fairly well correlated with the severity of the disease, although other liver function tests were more sensitive (Table III). Normal values were found in

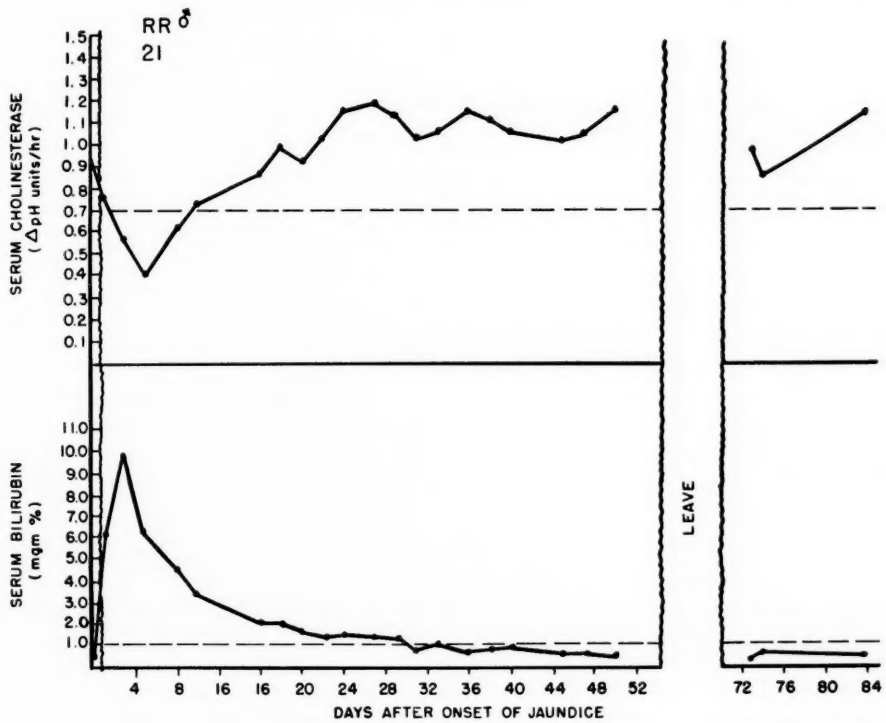

Fig. 2.—Serum cholinesterase and serum bilirubin in a typical case of acute viral hepatitis. In this case a determination was obtained by chance twenty-four days before the onset of jaundice (indicated by serrated line).

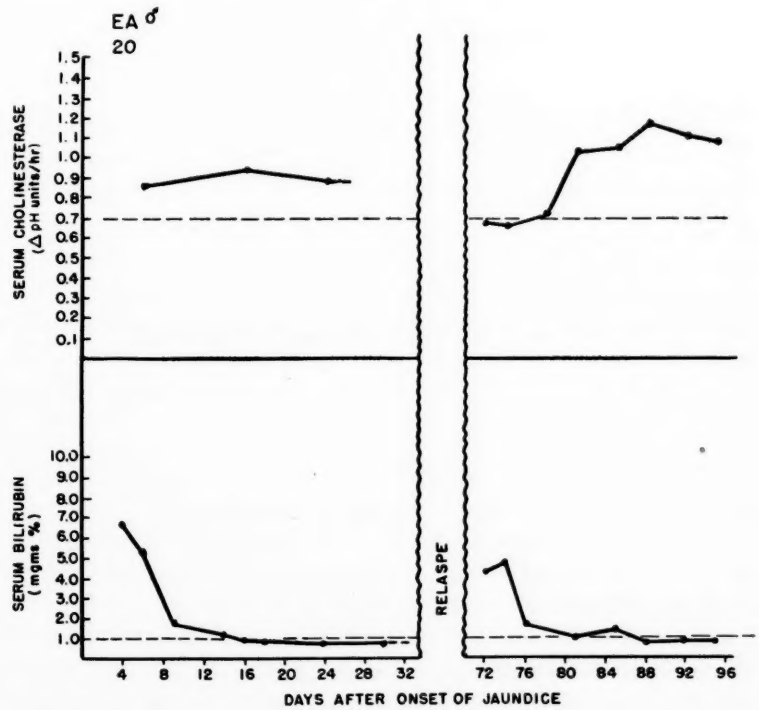

Fig. 3.—Serum cholinesterase and serum bilirubin in acute viral hepatitis with relapse.

TABLE I. CLASSIFICATION AND SELECTION OF GROUPS STUDIED

GROUP	NO. PRESENTING FOR STUDY	CRITERIA FOR SELECTION	NO. INCLUDED IN STUDY
Normal subjects	71	Apparently healthy blood donors and laboratory personnel.	71
Surgical patients	51	Patients without evidence of hepatic disease undergoing various surgical procedures, mostly minor. All cases had normal serum bilirubin values. In 7 cases, one or more flocculation test was abnormal.	51
Battle casualties	46	Major wounds and frostbite, often complicated by varying degrees of infection and malnutrition. (All exposed to serum hepatitis) but none had elevated serum bilirubins. In 12 cases, one or more flocculation test was abnormal.	46
Acute hepatitis	66	Majority were battle casualties. Included in this series only if followed from first week of jaundice until complete recovery. Diagnosed by typical clinical and laboratory findings. Liver biopsy provided additional confirmation in 22 of the 30 selected cases.	30
Chronic hepatitis	20	Diagnosed according to the following criteria: (1) Previous history of hepatitis, (2) Residual symptoms and/or laboratory evidence of activity, (3) Abnormal liver biopsy.	20
Obstructive jaundice	6	Biliary obstruction by stone or cancer; proved by surgery or autopsy in 5 cases, clinically in one.	6
Cirrhosis	21	Diagnosis by clinical and laboratory evidence and/or liver biopsy as indicated (Table III).	21
Fatty metamorphosis	6	Liver biopsy evidence of fatty infiltration without cirrhosis.	6

TABLE II. INCREASE IN SERUM CHOLINESTERASE WITH RECOVERY FROM ACUTE HEPATITIS (30 CASES)

VALUES (ΔpH UNITS/HOUR)

INITIAL	FINAL	INCREASE
0.65 ± .17*	0.94 ± .20	0.30 ± .19

*Mean standard deviation.

patients with mild degrees of fibrosis detectable only by biopsy (Cases 18 to 22), whereas the more severe cases usually showed lower values. Serial observations of cholinesterase were correlated with changes in the clinical condition in many cases, but the correlation was not consistent, particularly in the milder cases.

Fatty Metamorphosis.—Six cases of fatty metamorphosis demonstrated normal cholinesterase values.

DISCUSSION

There seems little doubt that the cholinesterase activity of the serum usually undergoes the changes previously described in parenchymal liver disease. The wide range of values encountered in the normal population and the even wider range observed in hospital patients without obvious liver disease tend to reduce its value as a diagnostic test. The test, however, is sufficiently simple to make practicable serial determinations in the same patient. One may then observe the trend and arrive at an approximate baseline value for each patient.

Our experience with the test in obstructive jaundice is too limited for firm conclusions but does suggest that it may not be precise in separating medical from surgical jaundice. Malnutrition[12] and malignancy[13] have been shown to depress the serum cholinesterase; in our small series, both of these factors were present in some cases rendering interpretation of individual values unusually difficult.

Despite these shortcomings serial serum cholinesterase determinations would be of considerable help to the physician if they reflected accurately the course of recovery from hepatitis and predicted the approach of the chronic phase of the disease.[4] While our data does show a rough parallel between an increase in cholinesterase activity and clinical improvement in acute hepatitis, this test does not reliably predict or reflect a relapse and it is usually normal in cases of proven chronic viral hepatitis.

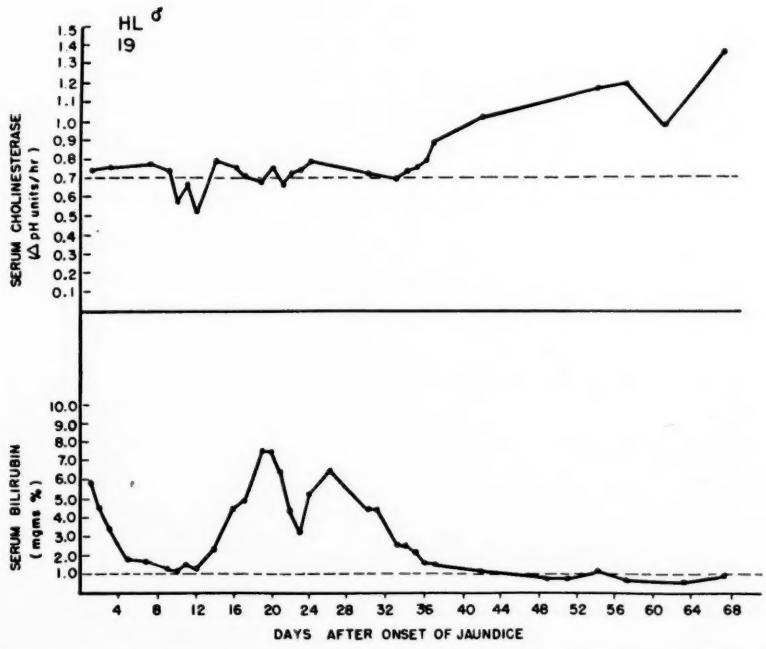

Fig. 4.—Serum cholinesterase and serum bilirubin in acute viral hepatitis with relapse.

The determination appears to mirror the clinical course most successfully in cases of hepatic cirrhosis. A low value in cirrhosis appears to be a grave prognostic sign. Alterations in the clinical condition of the patient are usually, but not always, correlated with corresponding fluctuations in serum cholinesterase activity. The test may be of assistance, therefore, in evaluating special therapeutic regimes in chronic liver disease and, possibly, in supplying additional evidence as to prognosis. Investigators studying protein synthesis in health and disease may find the technique of great value, since it appears to be well correlated with albumin synthesis.[14, 15] However, in the routine

TABLE III. SERUM CHOLINESTERASE IN CIRRHOSIS OF THE LIVER

CASE NO.	CHOLINESTERASE (ΔpH UNITS/HOUR)	BILIRUBIN (1' MG. %)	BILIRUBIN (TOTAL MG. %)	THYMOL TURBIDITY (UNITS)	CEPHALIN FLOCCULATION	ZINC SULFATE TURBIDITY (UNITS)	GAMMA-GLOBULIN (UNITS)	BSP (% RETENTION IN 45')	REMARKS
1	0.22	1.9	5.4	4.8	3+	14.5	8.4	–	Nutritional cirrhosis in deep coma. Died 8 days later. Autopsy.
2	0.27	0.57	2.5	3.6	3+	8.6	5.5	–	Nutritional cirrhosis in coma. Survived. Cholinesterase rose to .72. Biopsy.
3	0.28	0.16	1.2	3.4	1+	4.2	6.6	42	Nutritional cirrhosis with ascites. Improved but cholinesterase did not change.
4	0.33	0.55	1.7	1.1	0	3.7	–	51	Nutritional cirrhosis, moderate. Died 5 months later from acute alcohol poisoning. Autopsy.
5	0.35	0.33	1.4	7.0	3+	10.4	5.9	24	Biliary cirrhosis due to cholangitis. Moderate ascites. Biopsy.
6	0.37	0.81	2.1	1.7	3+	4.7	7.0	23	Biliary cirrhosis due to cholangitis. Biopsy. Cholinesterase rose to .86 without improvement.
7	0.41	2.4	4.6	3.4	0	4.5	2.6	–	Nutritional cirrhosis. Cholinesterase rose to 1.44 with clinical improvement. Biopsy.
8	0.43	0.39	1.0	1.2	0	2.5	2.9	18	Cirrhosis with severe ascites.
9	0.52	0.24	1.3	6.1	3+	8.4	4.5	17	Cirrhosis with varices.
10	0.56	0.50	2.1	11.9	4+	16.3	11.3	37	Nutritional cirrhosis, moderate. Biopsy.
11	0.61	0.93	2.0	1.4	0	1.0	–	22	Biliary cirrhosis due to stricture. Biopsy.
12	0.64	0.44	1.2	6.5	2+	11.9	5.4	23	Nutritional cirrhosis. Cholinesterase varied .33 to .66. Died with bleeding varices. Autopsy.
13	0.70	0.81	1.8	2.9	0	2.9	2.5	35	Biliary cirrhosis with recurrent cholangitis. Cholinesterase ranged to .48. Biopsy.
14	0.79	13.1	2.8	4.0	4+	2.2	2.5	–	Nutritional cirrhosis. Improved strikingly and cholinesterase rose to 1.15. Biopsy.
15	0.86	0.32	1.0	2.3	+	3.2	2.1	14	Nutritional cirrhosis, mild. No varices or ascites. Biopsy.
16	0.90	0.11	0.46	5.9	1+	7.5	–	13	Nutritional cirrhosis, mild. No varices or ascites. Biopsy.
17	0.97	0.06	0.38	2.5	0	2.2	–	30	Hemochromatosis with mild cirrhosis. Biopsy.
18	1.13	0.12	0.38	1.8	0	2.5	–	8	Nutritional cirrhosis, mild. No signs or symptoms. Biopsy.
19	1.23	0.15	0.74	11.9	0	8.1	4.7	–	Nutritional cirrhosis. No varices or ascites. Biopsy.
20	1.28	0.10	0.98	3.2	0	3.2	3.0	15	Post hepatitic cirrhosis with hepatosplenomegaly. No varices or ascites. Biopsy.
21	1.28	0.09	0.48	1.1	0	2.5	–	2	Post hepatitic cirrhosis. No signs or symptoms. Biopsy.

practice of medicine the serum cholinesterase test rarely provides the physician with information beyond that available by clinical means and conventional laboratory tests of liver function.

SUMMARY

Serum cholinesterase is usually decreased early in acute hepatitis and regularly increases as the patient convalesces. It does not always reflect a

relapse and is usually normal in chronic viral hepatitis. Patients with cirrhosis tend to have serum cholinesterase values which roughly parallel the severity of the disease. The wide range of values in normal subjects and patients without liver disease, the occurrence of low values in obstructive jaundice where malignancy or malnutrition is present, and its lack of discrimination of chronic viral hepatitis—all limit the usefulness of the cholinesterase determination as a practical test of liver function.

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THE SERUM PHOSPHOLIPID-CHOLESTEROL RATIO AS A TEST FOR CORONARY ATHEROSCLEROSIS

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A GREAT need exists for a clinical test which would detect active atherosclerosis. One of the first possibilities for such a test recently arose from the work of Kellner and associates[1] and by Ahrens and Kunkel.[2] These authors concluded that the size of the lipid droplets in serum is a function of phospholipid concentration, and that this effect is related to atherosclerosis.

Boyd[3] first noted the quantitative relationship between low serum phospholipid levels and lipemia, and stated that the concentration of serum phospholipids was the limiting factor in determining the lipemia and the clarity of the serum. Macheboeuf,[4] however, implicated a lipoprotein complex as responsible for maintaining lipids in clear serum or for determining lipid solubility.

A large amount of data has recently accumulated to indicate that chronic serum lipemia and serum hypercholesterolemia may initiate atherosclerosis.[5, 7-10] The interpretation of this data, however, has been opposed by other observers who note no correlation between chronic serum lipemia or serum hypercholesterolemia and the genesis of atherosclerosis.[6]

Kellner and co-workers,[1] in experimental studies, were able to decrease the incidence of atherosclerosis in rabbits by the intravenous injections of surface-active agents such as Tween 80 and Triton A 20. Although there was a resultant rise in serum cholesterol, there was a relatively greater rise in serum phospholipids which markedly increased the phospholipid/cholesterol ratio of the serum. Ahrens[11] recently presented additional evidence that a relationship appears to exist between the fixation of lipid in intimal cells and decreased phospholipid/cholesterol ratios. A marked increase in the incidence of atherosclerosis was found in a group of patients with nephrosis, hypothyroidism, and essential xanthomatosis, where the phospholipid/cholesterol serum ratio was less than one.

A significant study of the phospholipid/cholesterol ratio in a series of individuals who had experienced a myocardial infarction before the age of 40 years was recently reported by Gertler and his collaborators.[12] It was found that the coronary heart disease group had an appreciable increase in

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cholesterol/phospholipid ratio. The data reported here present the phospholipid/cholesterol ratios in a series of 124 unselected cases of coronary thrombosis as they were admitted to the Los Angeles County General Hospital.

MATERIALS AND METHODS

Sera were obtained from (1) a group of 92 normal control individuals who were in good health and symptom-free; (2) from a group of 124 patients admitted to the hospital because of a proved coronary thrombosis and myocardial infarction as shown by serial electrocardiograms, physical examination, and history; and (3) a group of 93 patients suffering from miscellaneous chronic diseases other than coronary thrombosis, such as pulmonary tuberculosis, osteomyelitis, nephritis, poliomyelitis, rheumatoid arthritis, ulcerative colitis, cirrhosis of the liver, and the like. Age and sex distributions of these groups is shown in Table I.

TABLE I. PHOSPHOLIPID TO CHOLESTEROL RATIOS

	CONTROL GROUP	CORONARY DISEASE GROUP	MISCELLANEOUS GROUP
Phospholipid/Cholesterol ratio:			
Mean ± S.E.	1.23 + 0.023	0.977 + 0.012	1.14 + 0.023
Range	0.73 – 1.79	0.42 – 1.75	0.60 – 1.65
Number of patients	92	124	83
Average age	45.4	54.5	51.6
Range	16-100	32-75	18-78
Males	46	79	44
Females	46	45	39

S.E. = Standard error of the mean.

The sera were all taken in the fasting state; total serum cholesterol determinations were determined by the Sperry-Schoenheimer procedure[13] or the Kingsley-Schaeffert method.[15] Phospholipids were determined as lipid phosphorus and expressed as lecithin (factor × 25) according to modifications of the method of Fiske and Subbarow.[16] The range of normal total serum cholesterol determinations was from 150 to 260 mg. per cent, whereas the normal phospholipid range was from 150 to more than 350 mg. per cent. In addition, determinations were made of the other lipid fractions such as the cholesterol esters, cholesterol esterases, total lipids, neutral fats, etc., but these data will be reported in a separate communication.

EXPERIMENTAL

The phospholipid/cholesterol ratios in each of the three groups are summarized in Table I and detailed in Table II. As noted in Table I, the range of phospholipid/cholesterol ratios for the Normal Control Group is from 0.73 to 1.79, with a mean of 1.23 plus or minus standard error of the mean 0.023. In the coronary disease group the phospholipid/cholesterol range was 0.42 to 1.75, with a mean of 0.977 plus or minus standard error 0.012. In the miscellaneous diseases group, the phospholipid/cholesterol range was from 0.69 to 1.65 with a mean of 1.14 plus or minus standard error of 0.023. The ratio of phospholipids to cholesterol in the serum of patients with coronary disease was significantly less than in normal controls and in a group of patients with various diseases other than coronary disease.

The mean total serum cholesterol determinations in the coronary disease group were the subject of previous reports by Morrison and associates,[7] and were found to be significantly elevated over those of the control group and the miscellaneous disease group.

TABLE II. DISTRIBUTION OF PL/C RATIOS

PHOSPHOLIPIDS: TOTAL CHOLESTEROL	PER CENT CONTROL GROUP	PER CENT CORONARY DISEASE GROUP	PER CENT MISCELLANEOUS GROUP
0.40-0.49	0.0	0.8	0.0
0.50-0.59	0.0	0.8	0.0
0.60-0.69	0.0	3.2	1.2
0.70-0.79	1.1	8.1	1.2
0.80-0.89	5.4	19.4	7.2
0.90-0.99	3.3	33.9	14.5
1.00-1.09	12.0	9.7	25.3
1.10-1.19	27.2	12.1	14.5
1.20-1.29	17.4	4.8	19.3
1.30-1.39	12.0	2.4	3.6
1.40-1.49	9.8	3.2	3.6
1.50-1.59	5.4	0.0	8.4
1.60-1.69	4.3	0.8	1.2
1.70-1.79	2.2	0.8	0.0

Table II gives a detailed breakdown of the phospholipid/cholesterol ratios in each of the three groups, arranged in fourteen increments of 0.1 per cent each from 0.40 to 1.79 inclusive. There is a shift in the frequency towards lower levels of phospholipids to cholesterol ratio in the coronary disease group as compared with the normal and the miscellaneous diseases group. Upon further examination of Table II it is seen that in the Coronary Disease Group, 66 per cent of the cases had ratios less than one. In the Control Group only 10 per cent of the cases had ratios less than one. In the Miscellaneous Disease Group 24 per cent of the patients had a ratio of less than one.

The cholesterol ester fractions were not found to be abnormal by the author and co-workers in previously reported groups of coronary disease and hence are not presented here.

In order to determine the validity of a single phospholipid/cholesterol determination, a series of ratio values were determined per individual in a

TABLE III. SERIAL STUDIES OF SERUM PL/C IN CORONARY DISEASE

CASE	ORDER OF TEST*	SERUM PHOSPHOLIPID†	TOTAL SERUM CHOLESTEROL†	PL/C RATIO
1. A. T., Male, Age 60 years	1	245	284	0.86
	2	258	270	0.93
	3	288	247	1.2
	4	280	297	0.81
	5	262	291	0.90
2. C. D., Male, Age 48 years	1	258	308	0.81
	2	312	364	0.86
	3	338	320	1.0
	4	352	285	1.3
	5	295	359	0.80
3. S. F., Female, Age 56 years	1	356	338	1.1
	2	318	357	0.90
	3	306	322	0.95
	4	320	329	0.97
	5	302	350	0 83

*Serial determinations made as fasting specimens from two to seven days apart.
†Milligram per 100 ml.

number of subjects in each of the three groups. In the Control Group, usually five although as many as six separate PL/C ratios in most cases these fluctuations tended to fall fairly consistently below the normal ratio of one.

Table III indicates PL/C values taken simultaneously on alternate days for five determinations. Table III also shows that in a group of patients with coronary disease a PL/C ratio may be normal, i.e., over one on one day and be found to be abnormal, or below one on three or four subsequent tests. This is in keeping with the disorder in lipid metabolism pointed out by Steiner,[5c] and Morrison[5d] and others to be present in coronary artery disease. It is also seen from Table III that repetition of PL/C determinations may be necessary to establish abnormal ratios.

TABLE IV. FAT TOLERANCE TESTS IN CORONARY DISEASE*

CASE	TEST HOUR*	SERUM PHOSPHOLIPID†	TOTAL SERUM CHOLESTEROL†	PL/C RATIO
1. Male, Age 42 years	F‡	150	189	0.79
	1	247	207	1.19
	2	200	191	1.04
	3	158	187	0.84
	4	160	185	0.87
2. Male, Age 48 years	F	238	285	0.83
	1	218	280	0.78
	2	168	250	0.67
	3	148	296	0.50
	4	200	298	0.67
3. Male, Age 51 years	F	256	230	1.11
	1	260	235	1.11
	2	260	230	1.12
	3	268	209	1.29
	4	282	211	1.33
4. Male, Age 61 years	F	270	283	0.95
	1	275	293	0.94
	2	266	333	0.80
	3	275	264	1.04
	4	305	263	1.15

*Breakfast taken contained two eggs, toast and butter, and a glass of milk.
†Milligram per milliliter.
‡F = Fasting specimen each test thereafter was taken hourly as designated.

Table IV shows the importance of determining phospholipids in the fasting state, since these values may be substantially influenced by the intake of fat in a meal. To determine this fact, four to five one-hourly determinations were made in a series of control and coronary disease patients, after they ate a breakfast containing butter, eggs, and cream. Under these circumstances marked fluctuations in PL levels may be observed, whereas in most cases the total cholesterol values are practically constant.

It is of interest to note that in Case 4 of Table IV, following breakfast, there is a fluctuation of 70 mg. of cholesterol between the readings taken at the two- and four-hour intervals. Although serum total cholesterol values do not tend to vary significantly in normal subjects, the author has found that they may fluctuate considerably in some patients with coronary disease, particularly after the patients have had a fat-containing breakfast such as that

used in this study. This may be an additional manifestation of disturbance in lipid metabolism, as evidenced by poor tolerance or serum cholesterol instability following a fat meal.

It is of clinical interest to remark here that recently two patients who presented themselves to the author for a routine physical check-up were found to have markedly abnormal PL/C ratios of 0.4 and 0.6, respectively. Although neither patient had symptoms referable to the heart, and in both cases electrocardiograms were normal, a typical coronary thrombosis with myocardial infarction occurred in each instance within one month after the tests. The possibility of the PL/C ratio as a "screening" test for coronary thrombosis might therefore be considered.

DISCUSSION

The determination of the PL/C ratio by the clinician as a single test to determine the presence of coronary disease is limited by the lipid fluctuations characteristic of coronary disease. However, when this test was combined by the authors with the saponin extraction method of Sobel and Morrison,[19] and with cholesterol esterase determinations, it was found that in most coronary disease cases two out of three of these tests indicated the detection of a disorder in the serum lipoprotein colloidal equilibrium. Upon further development, these three tests for coronary atherosclerosis may therefore hold promise as practical tools for the clinician and may thus be likened to the necessity for making more than one liver function determination in a test for liver diseases. Further studies on the coronary disease problem should be directed to determine if a combination of two or three tests reveals more completely the true serum colloidal state.

Previous studies by various investigators[17] including the author[18] have suggested that cholesterol and phospholipid metabolism are controlled to a great extent by the liver. This fact may be an additional consideration in the need for more than one test for eliciting biochemical reactions that represent functions of the liver.

SUMMARY AND CONCLUSIONS

The mean ratio of serum phospholipids to total serum cholesterol in patients with coronary artery disease was found to be significantly lower than the mean ratio in a normal control series and significantly lower in a series of patients with various diseases other than heart disease.

The majority of patients in a coronary disease group was found to have a serum phospholipid/cholesterol ratio of less than one. The majority of subjects in a normal control group and in a group of patients with various diseases was found to have a phospholipid/cholesterol ratio of more than one.

Variations in serum phospholipid/cholesterol ratios in the coronary disease group indicate that one negative PL/C ratio test did not rule out the presence of coronary artery disease since a subsequent test may be positive.

In coronary disease fluctuations occur in serum phospholipid levels and PL/C ratios as well as in the total serum cholesterol levels.

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THE TRANSFUSION OF HUMAN BLOOD PLATELETS

With a Note on the Transfusion of Granulocytes

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INTRODUCTION

IN A previous communication[1] it has been shown that following transfusion of polycythemic platelet-rich blood with multiple silicone-coated syringes a transfer of donor platelets into the recipient had been achieved. It was noted that by comparison with animal experiments the transfused platelets had a normal life span. Earlier attempts to transfuse platelets were also discussed.

The present study concerns itself with the application of this platelet transfusion technique to various thrombocytopenic states. An attempt has been made to evaluate the recovery of platelets in the recipients, the life span of transfused platelets, and the effect of these transfusions on the hemorrhagic state of thrombocytopenic individuals. The data to be presented further clarify the role of the platelets in hemostasis, and help to define the mechanisms of the thrombocytopenias in the patients studied. The value of this "platelet transfusion" technique in the therapy of thrombocytopenic states is pointed out. Observations on the transfusion of granulocytes are also included.

MATERIAL AND METHODS

1. *Direct Transfusion.*—Polycythemic donors, usually, with platelet counts between 500,000 and one million per cubic millimeter, were used. Normal donors were used only as noted in Table I. Donor and recipient were placed on beds side by side. An arquad coated 15-gauge, male hub stainless steel needle† connected to a silicone-coated syringe by a short piece of ⅛ inch polyvinyl Fenwal tubing was introduced into a vein of the recipient. After the vein had been entered and the needle taped in place, an assistant drew blood slowly into the syringe while venipuncture was being accomplished by means of similar equipment in the donor. Donor blood was collected into 50 ml. or 100 ml. silicone-coated syringes, with only occasional gentle traction on the plunger. As soon as a syringe had been filled with donor blood it was disconnected from the plastic tubing and replaced by a fresh syringe which was then filled in an identical manner. Meanwhile, the syringe filled with donor blood was attached to the plastic tubing of the recipient and the blood injected rapidly. This process was repeated until the desired amount had been transfused. Aseptic precautions were employed. Up to 1,500 ml. (taken from three normal donors) have been transfused in thirty to forty-five minutes. At a rate of about 500 ml. per fifteen to thirty minutes, each aliquot of blood was outside the body for an average of three to six minutes. No clotting took place in the syringes during this time. Small amounts of blood left in the syringes were usually found to be completely liquid thirty minutes later.

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†Kindly supplied by Fenwal Co., Ashland, Mass.

The recipient's antecubital vein was used whenever possible; but in children the external jugular vein, and in one instance, the femoral vein, was used. In two studies a No. 17 polyvinyl catheter was introduced into the femoral vein. The rate of blood flow obtained through the catheter was very much less than that obtainable with a 15-gauge needle.

Needles were coated with Armour's arquad 2C (dimethyl-dicocoammonium chloride)* by placing them in a 0.1 per cent solution of this material and heating the solution briefly to the boiling point. Syringes were coated with General Electric Dri-film 9977 in the following manner: the syringes were carefully cleaned with soap and water and then rinsed to remove all traces of soap. After thorough drying they were cooled to well below room temperature and were then placed into a glass cylinder into which silicone vapor was introduced by bubbling CO_2 through liquid silicone in a gas washing jar. A lid was placed over the glass cylinder for five to ten minutes, and the syringes were then removed. After being rinsed successively in tap water, distilled water, and finally pyrogen-free distilled water, they were sterilized by autoclaving. Immediately after use, they were rinsed with tap water and later in pyrogen-free distilled water. They were then reautoclaved and used again up to ten times before recoating.

2. *Platelet Counts.*—These were done on capillary or venous blood by the modified Reese-Ecker method.[2] Routinely two counting chambers were filled from one pipette, and the whole red cell counting area was counted in each chamber. When the platelet count was less than 40,000 per cu. mm., two pipettes were used and two counting chambers were filled from each pipette. Best results were obtained by using unfiltered light during the process of counting. Before being charged, counting chambers were carefully cleaned until the counting area was free of any artifacts which might be confused with platelets. By using only freshly filtered diluting fluid and doing counts shortly after collecting the specimens, the platelet blank was virtually reduced to zero. With platelet counts above 20,000, most duplicate determinations were within ± 10 per cent of the mean. Below this level the error increased so that with platelet counts of 5,000 a ± 20 per cent error had to be accepted.

3. *Bleeding Time,*[2] *Tourniquet Tests,*[2] *Clot Retraction,*[2] *and Prothrombin Consumption Tests*[3, 4] were done before and after transfusion in suitable cases. Great care was taken to duplicate exactly the technique in following any one patient.

4. *Bone Marrow Aspiration.*—Marrow was aspirated from the sternum, iliac crest, or tibia. Drops of the material obtained were placed on carefully cleansed glass slides which were then tilted to allow excess blood to run off while the bone marrow particles stayed in place. Smears were prepared from this "concentration" of marrow particles by dropping another slide on the preparation and pulling the slides apart. In this fashion highly cellular preparations were obtained. Megakaryocytes were not counted, but, from observation of the stained smears, their number was estimated as normal, markedly decreased, or markedly increased.

5. *Platelet Survival.*—Platelet counts were done before and within ten minutes after transfusion. In most instances counts were repeated three, six, and twenty-four hours later. After that, they were done daily.

The platelet count theoretically achievable by the transfusion was calculated as follows:

$$\text{Theoretical platelet count} = \frac{\left(\text{Volume transfused} \times \text{Donor's platelet count}\right) + \left(\text{Patient's blood volume} \times \text{Patient's platelet count}\right)}{\text{Patient's blood volume}}$$

The patient's pre- and posttransfusion counts were derived from capillary blood; the donor's platelet count was taken from a springe containing remnants of the blood that had been transfused. Blood volume was estimated from Gibson's tables[5, 6] on the basis of age in children and weight in adults. No correction was applied for anemia or organ enlargements. In patients who were not anemic, transfusion was preceded by phlebotomy of about the same quantity of blood as the transfusion. Several patients with severe anemia demon-

*Kindly supplied by Dr. Carl Walter.

PATIENT	CASE NO.	TRANSFUSION	PLATELETS × 10^3 BEFORE	PLATELETS × 10^3 AFTER	YIELD %	PLATELET SURVIVAL (DAYS)	HEMORRHAGIC SYMPTOMS BEFORE TRANSFUSION
Aplastic Anemia (Spleen Normal Size)							
G. P. ♀ 6 yrs.	1	1st	4	35	24	3	Generalized bleeding from mucous membranes and into skin prior to all but the third transfusion. Hematuria.
		2nd	4	128	49	--	
		3rd	48	165	47	5-6	
		4th	4	115	43	5-6	
		5th	4	38	18	1	
		6th	4	34	33	2	
		7th	4	60	36	1/8	
E. P. ♂ 2 yrs.	2		4	78	65	1-2	Generalized bleeding from mucous membranes, skin, and hematuria.
N. C. ♀ 43 yrs.	3		4	73	88	5-6	Petechiae and ecchymoses
P. Di C. ♂ 19 yrs.	4		10	57	55	6	None
Acute Leukemia (Spleen Moderately Enlarged)							
S. R. ♂ 4 yrs.	5		8	120	32	5	Hematuria, petechiae.
R. P. ♂ 4½ yrs.	6		20	100	50	Remission	None
Pancytopenia With Normal Marrow (Spleen Normal Size)							
F. B. ♀ 18 yrs.	7	1st	8	*	--	5	None
		2nd	10	141	88	5	None
		3rd	10	110	65	4	None
		4th	10	102	180	*	None
		5th	12	80	77	6	None
		6th	14	116	140	6	None
J. T. ♀ 21 yrs.	8	1st	8	90	69	6	Generalized; subarachnoid.
		2nd	6	84	65	5-6	Petechiae and ecchymoses.
		3rd	1	41	62	5-6	Menorrhagia and petechiae.
		4th	1.5	36	87	6	Menorrhagia and petechiae.
		5th	3	40	65	6	Menorrhagia and petechiae.
		6th	4	34	*	*	Menorrhagia and petechiae.
		7th	2	46	60	1	Menorrhagia and petechiae.
		8th	*	56	*	*	Intracranial bleeding.
A. M. ♀ 64 yrs.	9	1st	9	82	60	1/4-1/4	Bruising
		2nd	10	42	27	1/24-1/8	Bruising
Chronic Idiopathic Thrombocytopenia (Spleen Normal Size)							
H. J. ♀ 58 yrs.	10		7	75	73	1	Bruising
R. P. ♀ 29 yrs.	11		12	123	114	1	Bruising
R. W. ♂ 42 yrs.	12		10	60	94	2	None
B. McG. ♀ 46 yrs.	13		30	124	78	3-4	None
H. H. ♂ 23 yrs.	14		73	131	78	2-5	None
Acute, Self-limited Thrombocytopenia (Spleen Normal Size)							
M. S. ♀ 57 yrs.	15		2	5	0	0	Generalized
M. B. ♀ 14 yrs.	16		5	57	55	1/8	Menorrhagia
R. C. ♂ 7 yrs.	17		4	92	46	1/8	Bruising
N. D. ♀ 9 yrs.	18		4	95	36	1-2	Hematuria
L. G. ♀ 9 yrs.	19		3	43	24	1½	Hematuria
Thrombocytopenia with Massive Splenomegaly							
L. L. ♀ 6 yrs.	20		18	46	26	1	None
L. S. ♀ 41 yrs.	21		40	100	40	2/3	None
A. M. ♀ 66 yrs.	22		50	75	33	1/8	None
V. L. ♂ 58 yrs.	23		15	36	20	3	None

P. C. = Prothrombin Consumption; C. R. = Clot Retraction; B. T. = Bleeding Time; T. T. = Tourniquet Test.

*Not determined, not followed.

DURATION OF IMPROVEMENT (DAYS)	HEMORRHAGIC TESTS		REMARKS
	CHANGED	UNCHANGED	
5	P. C., C. R.		Technique unsatisfactory.
8	P. C., C. R.		Third transfusion given two days after second transfusion. Platelet count still elevated.
5			
2			
¼			All transfusions given over a period of one month.
None. Patient died.			
2; Hematuria recurred after 5 days		None done	
No fresh lesions for 6-8 days		None done	Normal donors.
--------	B. T. > 10 to 8 T. T. 2+ to 0		Normal donors.
No fresh petechiae for 5 days. Hematuria ceased.		None done	
--------		None done	
--------		None done	Transfusions given over period of six months.
--------		None done	
--------		None done	
--------		None done	Splenectomy one day after third transfusion.
--------		None done	
--------		None done	
7	B. T. 10 mins. before, 6½ mins. 12 hrs. after 1st transfusion		Except for 2nd and last transfusion, transfusions were given at monthly intervals on 4th to 7th day of successive menstrual periods.
7			
Vaginal bleeding ceased within 12 hours. No fresh petechiae for 7 days.			
Improved	T. T. 4+ → 0		All transfusions given over period of six months.
Patient died 24 hours after transfusion			Transfusions 3, 4, 5, 6, 7 from normal donors.
None		B. T.	
None			
None	?P. C., C. R. 0→Normal	B. T., T. T.	
None	C. R. 0→Normal	B. T., T. T., P. C.	Splenectomy 17 years before transfusion
--------	T. T. and B. T. Normal before transfusion		Splenectomy 18 months before transfusion
--------			

None		None done	Splenectomy 2 weeks before transfusion
3	T. T., 4+ → 0 B. T. 15 mins. → 8		
None	B. T. 15 mins. → 3		Two molars removed immediately after transfusion without excessive bleeding.
Ceased within 12 hours	T. T. 4+ → 0, P. C. B. T. 15 → 3½ mins. C. R. 0 → Normal		
Remission 24 hours later	T. T. 4+ → 0 T. T. 4+ → 12 hours later		
--------		None done	Banti's disease
--------		None done	Tuberculous splenomegaly
--------		None done	Malignant lymphoma
--------		T. T., 3+ before and after	Chronic melocytic leukemia

strated marked hemoconcentration by the time a transfusion of 500 to 700 ml. was ended. For these reasons theoretical platelet counts were calculated on the assumption that the patient's total blood volume had remained unchanged as a consequence of the transfusion.

After a recipient had received a direct transfusion, a percentage platelet yield was calculated in the following manner:

$$\text{Percentage platelet yield} = \frac{\text{Highest posttransfusion platelet count} - \text{Pretransfusion platelet count}}{\text{Theoretical platelet count} - \text{Pretransfusion platelet count}}$$

The ten-minute platelet count was usually the highest posttransfusion count. In the few instances in which the three-hour platelet count was higher, this figure was used as the basis for determining the yield.

Platelet survival was determined by noting the return of the platelet count to or below pretransfusion levels. The platelet count frequently dropped below pretransfusion levels, but the first platelet count which was no longer above pretransfusion levels was taken as the end point.

6. *Granulocyte Counts.*—These were obtained by doing white counts with two pipettes and four counting chambers, and multiplying by the percentage of granulocytes seen in 200 nucleated cells on a stained blood smear. Theoretical granulocyte levels, granulocyte yields, and survival were determined in the same manner as for platelets.

7. *Cases Studied.*—

A. Aplastic Anemia (Cases 1, 2, 3, and 4): In Cases 1 and 2 the bone marrow was aplastic with practically no megakaryocytes, as judged by repeated bone marrow aspirations, surgical biopsy, and eventually autopsy. In Case 1 the thrombocytopenia was of six months' duration. No cause for this bone marrow aplasia was discovered. In Case 2 bone marrow aplasia was apparently congenital. In Cases 3 and 4 bone marrow studies were not done, but the bone marrow was assumed to be aplastic because transient pancytopenia followed therapy with triethylene melamine* which is known to produce bone marrow aplasia in some patients. The spleen was not enlarged as determined by roentgenogram and palpation.

B. Acute Leukemia (Cases 5 and 6): The bone marrow was completely replaced by leukemic infiltration on aspiration and biopsy. Megakaryocytes were absent. Thrombocytopenia was of six months' duration in Case 5 and of five months' duration in Case 6. The spleen was moderately enlarged.

C. Pancytopenia with Normal Bone Marrow (Cases 7, 8 and 9): In Case 7 repeated bone marrow aspiration revealed low normal numbers of megakaryocytes. The duration of the thrombocytopenia was two years at the time of the first direct transfusion. In Case 8 the duration of the disease was two months at the time of the first transfusion. Sternal marrow aspiration at this time revealed normal numbers of megakaryocytes. No further bone marrow aspirations were done because of the extreme bleeding tendency. At autopsy, six months later, the bone marrow was found to be markedly hypoplastic, but megakaryocytes appeared to be the least affected of the marrow constituents. In Case 9 the disease was of one year's duration at the time of the first transfusion. Repeated sternal aspirations revealed mature megakaryocytes to be entirely lacking, but immature forms were present in greater numbers than usual. In Cases 7 and 9 erythropoiesis and myelopoiesis were extremely active. The spleen was not enlarged in any of these patients.

D. Chronic Idiopathic Thrombocytopenia (Cases 10, 11, 12, 13, and 14): Repeated bone marrow aspirations yielded normocellular preparations with normal to increased numbers of megakaryocytes. The disease was of at least two years' duration. Cases 11 and 12 had

*Patients 3 and 4 had normal blood values before treatment with triethylene melamine. Patient 3 was treated with this drug for Hodgkin's disease with only localized lymph node involvement, and patient 4 had a transitional cell carcinoma of the spine without apparent metastases.

splenectomy without relief seventeen and one and one-half years ago, respectively. In Cases 10, 13, and 14 the spleen was present but not palpable. All patients were asymptomatic at the time transfusion was given.

E. Acute Self-limited Thrombocytopenia (Cases 15, 16, 17, 18 and 19): Bone marrow aspirations shortly before transfusion yielded normocellular preparations with normal numbers of megakaryocytes. The disease was of short duration (a matter of weeks or months at the most). It was idiopathic in Cases 15 and 18, and followed mumps in Case 16, chicken pox in Case 17, and German measles in Case 19. All have since experienced spontaneous clinical and hematologic remission.* Case 15 had undergone splenectomy without benefit two weeks before transfusion. In the other patients the spleen was not palpable. All patients had some evidence of a bleeding tendency at the time of transfusion, although this varied markedly in extent and severity.

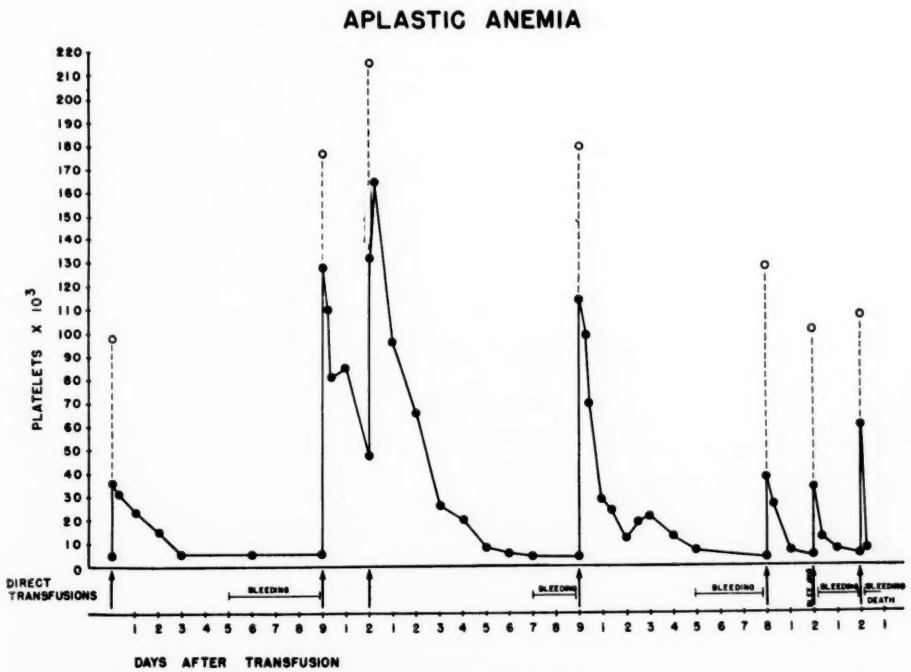

Fig. 1.—Case 1. Results of repeated "platelet transfusions." (– – –○, theoretical platelet counts: —●—, observed platelet counts.)

The first transfusion was technically unsatisfactory. No difference in yield between the second and third transfusions although prior to the third transfusion the patient did not have severe thrombocytopenia. The life span of the platelets was six to seven days originally (third transfusion), but repeated transfusions were decreasingly effective.

F. Thrombocytopenia with Massive Splenomegaly (Case 20, Banti's syndrome; Case 21, tuberculous splenomegaly; Case 22, malignant lymphoma; and Case 23, chronic myelocytic leukemia): Cases 20, 22, and 23 had normal bone marrow aspirations with normal numbers of megakaryocytes. Case 21 (tuberculous splenomegaly) had hypoplastic bone marrow by aspiration. All but the patient with malignant lymphoma underwent splenectomy, which was followed by a rise in the platelet count to normal within two days. The elevation of the platelet count was maintained in Case 20 until the present (ten months later), in Case 21 until death (three months later), and in Case 23 for only one month, at which time the disease changed to myeloblastic leukemia.

*Remission began the day after transfusion in Case 19 and four days after transfusion in Case 18.

RESULTS

The results of direct transfusions on the platelet count, hemorrhagic tests, and hemorrhagic symptoms of the recipients are tabulated in Table I.

Of forty-two transfusions given to twenty-three patients, only one failed to raise the platelet count significantly. From 20 to 100 per cent of the platelets given were recovered in the recipients. Platelet counts were raised by as much as 130,000 per cubic millimeter. Highest yields and longest platelet survivals were noted in the patients with aplastic anemia (Fig. 1), acute leukemia, and pancytopenia with normal bone marrow (Fig. 2). Lowest yields and shortest platelet survivals were seen in acute thrombocytopenia (Fig. 3) and in the thrombocytopenias associated with massive splenomegaly (Fig. 4).

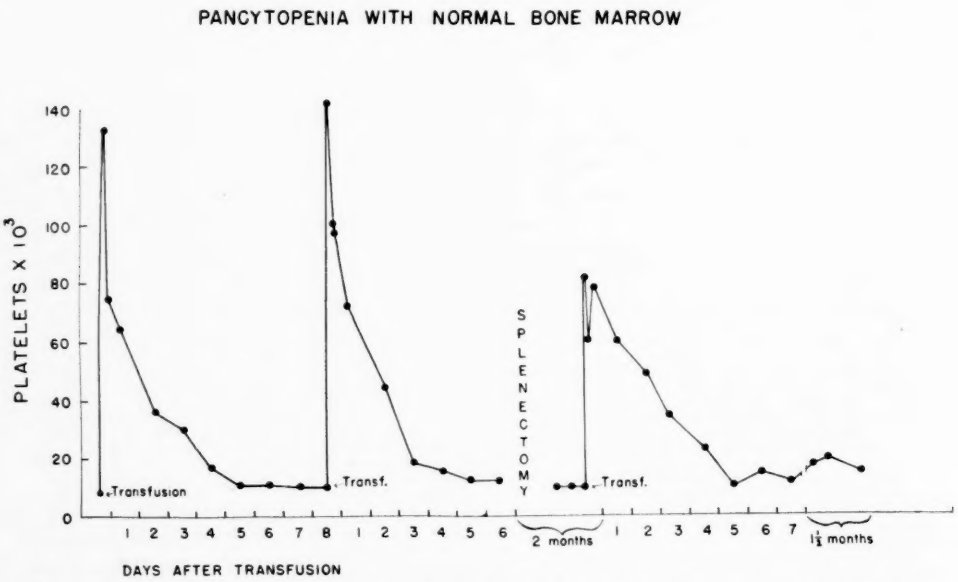

Fig. 2.—Case 7. Results of "platelet transfusions." Platelet survival is the same as in aplastic anemia and was not altered following splenectomy.

In chronic idiopathic thrombocytopenia yields were uniformly high, but platelet survival varied. It was never as short as in acute thrombocytopenia or thrombocytopenia with massive splenomegaly, but platelet survival was never as long as in aplastic anemia (Fig. 5). Three patients (Cases 1, 7, and 8) were transfused repeatedly. Two of these showed decreasing yields, shortened platelet survival, and lessening effect on the hemorrhagic state with successive transfusions (Fig. 1).

Clinically, the most striking result of direct transfusions was the dramatic cessation of oozing from the gums and from the site of needle punctures and bone marrow aspirations (Cases 1, 2, and 8). Wounds which had been oozing for several days and in which bleeding could not be controlled by pressure dressings stopped bleeding while transfusions were still being given. Only Case 15, in whom no rise in platelet count was obtained, failed to show immediate cessa-

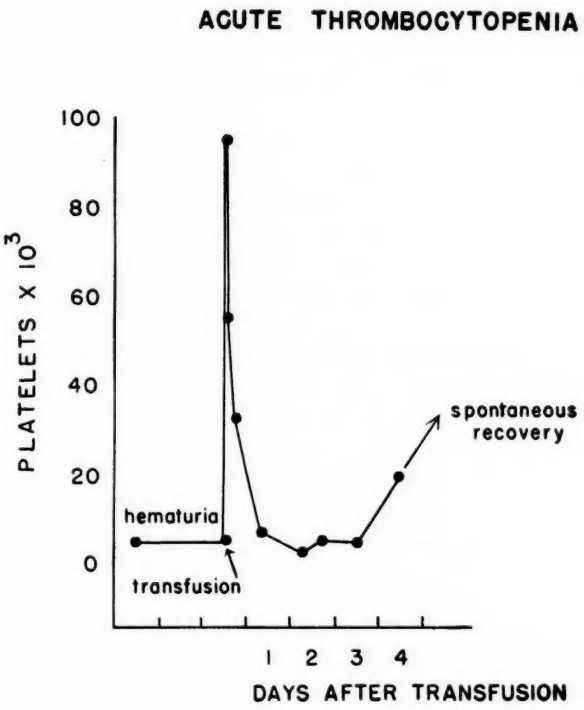

Fig. 3.—Case 18. This case showed the longest platelet survival observed in acute thrombocytopenia. Platelet survival is much shorter than in aplastic anemia, indicating platelet destruction.

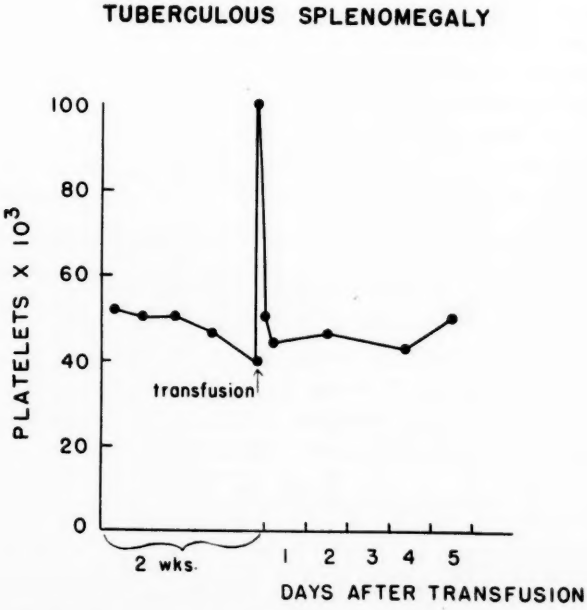

Fig. 4.—Case 21. Platelet survival is much shorter than in aplastic anemia, indicating rapid platelet destruction.

tion of bleeding. Cessation of hematuria and cessation or amelioration of vaginal bleeding within twenty-four hours after transfusion occurred in all patients in whom such findings were present (Cases 1, 2, 5, 8, 16, 18, and 19). Since blood was presumably still present in the bladder or the uterus after bleeding had ceased, cessation of apparent bleeding from these sites was less dramatic than that from skin wounds.

In Case 8 it is almost certain that intracranial bleeding was stopped by a direct transfusion. This patient was admitted with a stiff neck and xanthochromic spinal fluid which contained crenated red cells. During the next three days she became increasingly stuporous, and twelve hours before transfusion

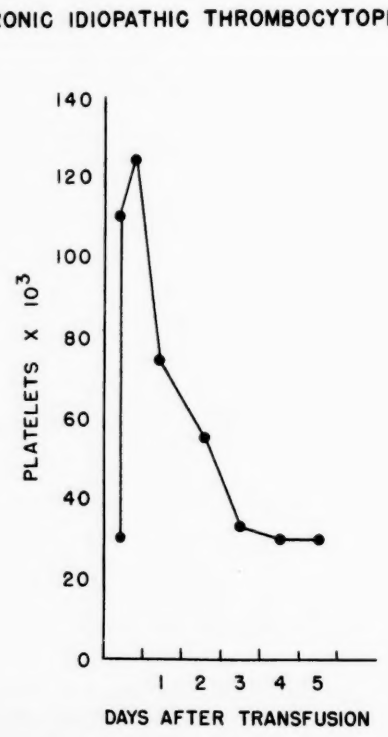

Fig. 5.—Case 13. This case showed the longest platelet survival obtained in chronic idiopathic thrombocytopenia. Platelet survival is shorter than in aplastic anemia but longer than in acute thrombocytopenia.

had a convulsion associated with frankly bloody spinal fluid. When transfusion was begun she was comatose, and was bleeding from the gums, needle puncture sites, and uterus. Superficial bleeding ceased while the transfusion was being given. Within twelve hours she had become conscious and rational, and vaginal bleeding had ceased.

When transfused platelets remained in the circulation for at least four days, no new petechiae and ecchymoses appeared during the life span of the transfused platelets. With platelet survivals of less than one day this could not be evaluated.

Bleeding from needle punctures did not resume after the platelets had dropped to their previously low levels. Similarly hematuria and menorrhagia were often relieved beyond the life span of the transfused platelets. Oozing from the gums and fresh petechiae and ecchymoses, however, usually reappeared at about the time the platelet count returned to its original level.

Patients in whom hemorrhagic symptoms improved showed marked improvement in bleeding time, tourniquet test, clot retraction, and prothrombin consumption tests whenever such tests were performed. As the platelet count fell the results of these tests, too, became progressively abnormal.

Among the patients who had no or minimal hemorrhagic symptoms, hemorrhagic tests were done in five (Cases 4, 9, 10, 11, and 23). Only one of these (Case 4) showed normal platelet survival. In this patient a significant change in bleeding time and tourniquet test occurred. No effects on bleeding time and tourniquet test were noted in Cases 9, 10, 11, and 23 who showed rapid platelet destruction.

Eight of the transfusions were followed by chills and fever, but none was followed by hemolytic transfusion reactions. High yields, normal platelet survival, and improvement of the hemorrhagic state occurred despite these reactions.

DISCUSSION

Although in the present study it was impossible to differentiate between the platelets of the donor and those of the recipient, the immediate rise in platelet count following direct transfusion suggests that an actual transfer of platelets was accomplished. In the few instances in which the three-hour platelet count was higher than the ten-minute count, the rise may have been due to incomplete mixing of the transfused blood with that of the recipient, or to further hemoconcentration. Since the percentage yields are calculated on the basis of blood volume estimated by age or weight and on the assumption that the blood volume remained unchanged as the result of transfusion, only an approximation of the true yield was obtained. The apparent blood volume was evidently overestimated in splenectomized individuals, since in these patients yields of over 100 per cent were observed. Likewise in patients with massive splenomegaly blood volume estimates on the basis of age or weight, and therefore yields, were probably meaningless. Except for such patients, yields were 50 to 90 per cent in those with longest platelet survival. In patients with extensive bleeding, yields were not significantly different from those in patients without bleeding. When two transfusions were given in rapid succession, the yield in the second was no higher than that of the first (Fig. 1). It would thus appear that in the process of stopping hemorrhage, platelets are utilized in smaller numbers than the error of the yield. The possible utilization of platelets by "platelet-starved tissues" must also be relatively small.

The data available at present are insufficient to evaluate the possible presence or absence of naturally-occurring platelet antibodies of the type seen in the human ABO system, but the progressive decrease in platelet survival in two of the three patients who received repeated direct transfusions suggests that platelet destruction may occur as a consequence of repeated direct transfusions.

Direct transfusions had been given to Case 9 prior to her coming under observation and probably also to Case 2, hence the short platelet survival in these two patients may have been due to the previous transfusions. Since no data are available to indicate whether stored blood transfusions are capable of sensitizing against platelets, platelet survival studies in patients with previous transfusion therapy must be interpreted with care, if survival is short. Case 11 was the only other patient with short survival who had received many stored blood transfusions prior to direct transfusion.

The possibility that platelets of polycythemic donors were themselves abnormal and subject to unusually rapid or slow destruction was excluded as far as possible by noting that the platelets of each polycythemic donor survived normally (see next paragraph) on at least one occasion. Furthermore, in three patients the survival of the platelets from normal donors was the same as that of platelets from polycythemic donors.

It seems reasonable to assume that in aplastic anemia failure of platelet formation due to lack of megakaryocytes is the only cause of the thrombocytopenia. Hence, the life span of platelets transfused into patients with aplastic anemia was taken to represent the normal life span of platelets in this study. Excluding the patient who had received previous direct transfusions, excellent agreement for the life span of platelets was noted in the three patients with aplastic anemia. From these observations the life span of human platelets was from five to six days. This life span is in excellent agreement with the results of animal experiments[7] (see also[1] for further references).

In acute thrombocytopenia rapid platelet destruction was evident not only from the short survival of transfused platelets (less than three hours in three patients) but also from their low recovery. The short platelet survival in these cases is comparable to the rapid development of thrombocytopenia in cases of sedormid[8, 9] and quinidine purpura.[10, 11] Thus rapid platelet destruction apparently occurs in all types of acute thrombocytopenia whether postinfectious, idiopathic, or due to drug sensitivity.

Ackroyd's observations[12] suggested that in several cases of sedormid purpura excessive platelet lysis occurred in vitro. Evidence for a plasma factor in acute idiopathic thrombocytopenia which causes platelet destruction in vivo was obtained by Harrington and associates[13] He demonstrated that transfusion of 500 ml. of blood from patients with acute thrombocytopenia caused severe thrombocytopenia in normal recipients within three hours. It appears likely that the rapid destruction of transfused platelets in the cases of acute thrombocytopenia in this study may be due to this factor.

Following direct transfusion of patients with chronic idiopathic thrombocytopenia, platelet yields were higher than in acute thrombocytopenia, but the life span of transfused platelets was variable. Although the life span of transfused platelets in chronic idiopathic thrombocytopenia was never normal compared to that observed in patients with bone marrow aplasia, it was not as short as in acute thrombocytopenia. These observations suggest that platelet destruction occurs in this disease also, but at a slower rate. This slower rate of destruc-

tion may explain the somewhat higher basal platelet counts of these patients* and the instances in which blood from patients with idiopathic thrombocytopenia failed to produce thrombocytopenia in normal recipients.[13] In both acute and chronic thrombocytopenia, increased platelet destruction occurred also in splenectomized individuals. Thus the spleen is not essential for platelet destruction in these diseases, nor did splenectomy prolong the life span of transfused platelets, when they survived normally prior to splenectomy (Fig. 2).

Among the three patients with pancytopenia, the life span of transfused platelets was initially normal in two. Since megakaryocytes were normal in number, the absence of platelet destruction suggests that in these two patients thrombocytopenia may be due to failure of platelet formation by the megakaryocytes or their release from the marrow. In the third patient (Case 9) transfused platelets were rapidly destroyed. Since this patient had received direct transfusions previously, platelet destruction may have been the result of these transfusions and not necessarily present at the onset of the thrombocytopenia.

The very short platelet survival in the four patients with massive splenomegaly indicated rapid platelet destruction. This might be in keeping with Doan's concept[14] of platelet sequestration by the spleen, since no evidence of a platelet-destroying plasma factor has been obtained in cases of this type.

The short survival of transfused platelets in acute thrombocytopenia, chronic idiopathic thrombocytopenia, and thrombocytopenia with massive splenomegaly suggests that platelet destruction is one of the possible mechanisms for thrombocytopenia in these diseases. Dameshek and Miller,[15] however, concluded from their studies on smears of aspirated marrow that in acute and chronic idiopathic thrombocytopenia platelet formation was markedly decreased or even absent. Thus, the possibility exists that platelet destruction in these and other thrombocytopenic states is merely an incidental finding and that no platelets are released to be destroyed by factors in the plasma or elsewhere. In the two patients with pancytopenia with normal bone marrow, however, the absence of platelet destruction makes it appear most likely that failure of platelet formation by the megakaryocytes is the cause of the thrombocytopenia.

Since direct transfusions were capable of causing cessation or at least amelioration of bleeding in thrombocytopenic individuals, it is clear that bleeding could not have been primarily due to a vascular difficulty or a circulating anticoagulant, although such factors might aggravate an already existing bleeding tendency. The excellent correlation between platelet survival and the duration of improvement (Fig. 1) suggests that improvement of the hemorrhagic state was due to transfused platelets and not to some other factor in the transfused blood. Moreover, several of the patients had failed to respond to fresh bank blood (less than twelve hours old) and to serum.

TRANSFUSION OF GRANULOCYTES

The patients with aplastic anemia (Cases 1 to 4), pancytopenia (Cases 7 to 9), and three of the patients with massive splenomegaly (Cases 20 to 22),

*Assuming that transfused platelets are handled by the body in similar fashion to the patient's own platelets.

TABLE II. RESULTS OF GRANULOCYTE TRANSFUSIONS

PATIENT	CASE NO.	GRANULOCYTES × 10^2 BEFORE	GRANULOCYTES × 10^2 AFTER	YIELD (%)	SURVIVAL HOURS	REMARKS
E. P.	2	6	4	0	0	Polycythemic donor with 9,000 granulocytes per cu.mm.
N. C.	3	2	5.5	35	<24	Normal donors
P. di C.	4	6.5	12.5	33	>6<24	Normal donors
F. B.	7	5 (1st transfusion)	18	12	<24	Polycythemic donor with 55,000 granulocytes per cu.mm.
		4 (2nd transfusion)	16	86	>6<24	Polycythemic donor with 12,000 granulocytes per cu.mm.
		18 (6th transfusion)	80	69	>6<24	Polycythemic donor with 55,000 granulocytes per cu.mm.
J. T.	8	2 (4th transfusion)	8.5	27	<24	Normal donors
A. M.	9	10	11	0	0	Polycythemic donor with 13,000 granulocytes per cu.mm.
A. M.	22	19	25	32	<3	Polycythemic donor with 55,000 granulocytes per cu.mm.

had sufficiently low granulocyte counts to permit studies on transfused granulocytes. Such studies were performed only in Cases 2, 3, 4, 7, 8, 9, and 22 (Table II). Cases 2, 9, and 22, who showed rapid platelet destruction, had no significant rise in granulocytes, or granulocyte survivals of less than three hours. In the patients who showed normal platelet survival, granulocyte yields between 12 and 86 per cent were obtained. The life span of transfused granulocytes in the circulation of these patients was longer than six hours but less than twenty-four hours. Though hourly counts were not done to evaluate these data more clearly, they are in agreement with the values obtained by Lawrence[16] in cats made leukopenic by irradiation. No ready criteria were available to evaluate any therapeutic effect of these "granulocyte transfusions."

SUMMARY AND CONCLUSIONS

Forty-two transfusions of polycythemic or normal blood without anticoagulants were given to thirty-five thrombocytopenic individuals by means of multiple silicone-coated syringes.

Such transfusions were found to be effective in temporarily raising the platelet counts and in causing cessation of spontaneous bleeding in thrombocytopenic individuals.

Transfused platelets remained in the circulation for five to six days in patients with bone marrow aplasia. This period was taken as the normal life span of transfused platelets in this study.

Rapid destruction of transfused platelets occurred in acute thrombocytopenia and in thrombocytopenia with massive splenomegaly. In chronic idiopathic thrombocytopenia platelet destruction also occurred but was less rapid. Transfused platelets survived normally in two cases of pancytopenia with normal bone marrow and in one case of acute leukemia.

In acute thrombocytopenia, chronic idiopathic thrombocytopenia, and in thrombocytopenia with massive splenomegaly, excessive platelet destruction is a possible mechanism of the thrombocytopenia. In two cases of pancytopenia with normal bone marrow and in one case of acute leukemia, no evidence for such a mechanism was obtained.

Repeated direct transfusions may cause progressive shortening in the survival of transfused platelets.

The circulating life span of transfused granulocytes was longer than six hours but less than twenty-four hours.

We wish to express our gratitude to Drs. L. K. Diamond, B. Alexander, and W. B. Castle for allowing us to study their patients, and to Drs. H. A. Avery, F. S. Bigelow, W. H. Borges, J. F. Desforges, R. Goldstein, and W. H. Zinkham for thier assistance in these studies.

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THE EFFECT OF CORTISONE AND ACTH IN LEUKEMIA

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IT HAS been known for some time that the secretions of the adrenal cortex influence the hematopoietic tissues, particularly the lymphatic tissue. (Selye[1] and White.[2]) In 1944 Heilman and Kendall[3] reported that 11-dehydro-17-hydroxycorticosterone (cortisone) was effective in halting the growth and causing involution of established lymphoid tumors of mice as well as preventing their successful transfer. They pointed out that this effect was not as pronounced in male mice and was much more effective in inhibiting tumor growth in young or female animals. They postulated that the mechanism of this inhibition might be that "the rapid catabolism induced by compound E surpasses the capacity of the tumor cells to respond within safe physiologic limits, with the result that these cells are destroyed." In the same year Murphy and Sturm[4] reported on the effects of desoxycorticosterone acetate, Eschatin (a mixture of adrenal cortical hormones in oil), and pituitary adrenotropic hormone upon transplanted rat leukemia. They found that these substances markedly improved the survival rate following intraperitoneal inoculation of leukemic cells. In the past six years additional work (Diller,[5] Lewis,[6] and Law[7]) has tended to substantiate the observation that the adrenal steroids exert a retarding action on the growth of lymphoid tumors of mice and rats.

Attempts to influence the course of malignant disease in man stem naturally from such animal experimentation. The remarkable clinical and metabolic effects of the pituitary adrenocorticotrophic hormone (ACTH) and cortisone have encouraged their use in various forms of human neoplasia. Although neither agent has been effective in carcinomata, each has proved of some benefit in the treatment of lymphatic tumors and leukemia.[8-18] The results, unfortunately, have not been uniform, and at present appear unpredictable. Remissions have been induced in some, in others the disease has been held in check temporarily, while a small group has been completely refractory. Further knowledge of the mechanism of action of the adrenal steroids, their proper dosage, and manner of administration are much to be desired. While it is unlikely that the clinical

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approach can yield more than a partial and imperfect solution to these problems, sufficient experience may uncover clues which will enable the physician to select and treat his patients more intelligently.

Studies on ten cases of leukemia form the basis of this report. Six of these were treated with cortisone alone, three with ACTH alone, and one with both cortisone and ACTH (all given intramuscularly). One patient with acute leukemia who received cortisone was the subject of an extended metabolic investigation, the results of which are reported in detail. The clinical observations in the remaining nine patients have been summarized briefly.

PROCEDURE AND METHODS

All patients were hospitalized and under continuous observation during the course of study. U.S. Bureau of Standards equipment was used in carrying out the hematologic studies.

Case 1 (K.N.) was the subject of a metabolic balance study during cortisone therapy. The dietary intake was computed from analysis of sample menus prepared at intervals of five days. During the first fifty days the average values were found to be: Ca 1.89 Gm., P 1.98 Gm., N 15.58 Gm., Na 182.7 meq., and K 104.6 meq. Calories were estimated as 2,675, using standard dietary tables. Because of the patient's voracious appetite, the diet was changed at the end of the tenth period to one having the following daily composition: Ca 2.41 Gm., P 2.53 Gm., N 19.98 Gm., Na 233 meq., K 121.4 meq., and 3,432 calories. These average values have been plotted in the figures pertaining to the metabolic balances (see Figs. 1, 2, 3, and 5). Cortisone was administered intramuscularly in two daily doses of 75 mg. each (150 mg. per day) during periods 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and 18 (a total of sixty-five days during which 9.75 Gm. were given). The urine was analyzed daily for sodium, potassium, phosphorus, and nitrogen; urine collections were pooled for five-day periods, and the analyses repeated as a check on the daily values. Stool collections were pooled in five-day periods and analyzed for the same elements as urine. The average values for fecal excretion of the various elements have been plotted in Figs. 1, 2, 3, and 5. It will be noted in these figures that the fecal excretion was averaged for periods 1 through 10, when the patient was on Diet No. 1, and periods 11 through 18, when the patient was on Diet No. 2. Values for five-day period stool analyses were not plotted since it is felt that difficulties encountered in accurately separating stools into five-day periods preclude the recording of other than average data. Emesis and dietary rejects were collected and analyzed. Determinations of blood chemistry were carried out and included analyses for sodium, potassium, calcium, phosphorus, total serum proteins, albumin, globulin, and nonprotein nitrogen. Daily hematologic observations included determination of packed red-cell volume, red blood cell count and hemoglobin, total, and differential leukocyte counts. For the sake of brevity, these determinations have been omitted from the paper except when fluctuations were of significance.

Analytical Methods.—Aliquots of diet, feces, urine, and emesis were ashed in a muffle furnace at a temperature not exceeding 450° C., the ash dissolved in concentrated hydrochloric acid and subsequently diluted to a volume of 200 ml. with distilled water. Sodium and potassium determinations were performed directly on diluted specimens of urine and blood serum, using the Beckman flame photometer. With the exception of nitrogen, the other substances were determined upon the ashed filtrates. Urinary, fecal, and dietary calcium were determined by the gravimetric method of Washburn and Shear[19]; serum calcium by the method of Van Slyke and Sendroy.[20] Phosphorus was determined by a modification of the colorimetric method of Fiske and Subbarow,[21] and the results so obtained were compared from time to time with the gravimetric procedure of Washburn and Shear[19]; nitrogen by the Hiller, Plazin, and Van Slyke modifications of the Kjeldahl procedure[22]; chlorides by modifications of the Volhard method[23-25]; plasma proteins by a modification of the Howe sodium sulfate method[26]; nonprotein nitrogen by the method of Koch and McMeekin.[27]

RESULTS AND DISCUSSION

For the purpose of clarity this section will be divided into two parts: (a) Metabolic results in a single case of acute myeloblastic leukemia on whom detailed balance studies were conducted, and (b) clinical results in nine cases of leukemia receiving these drugs.

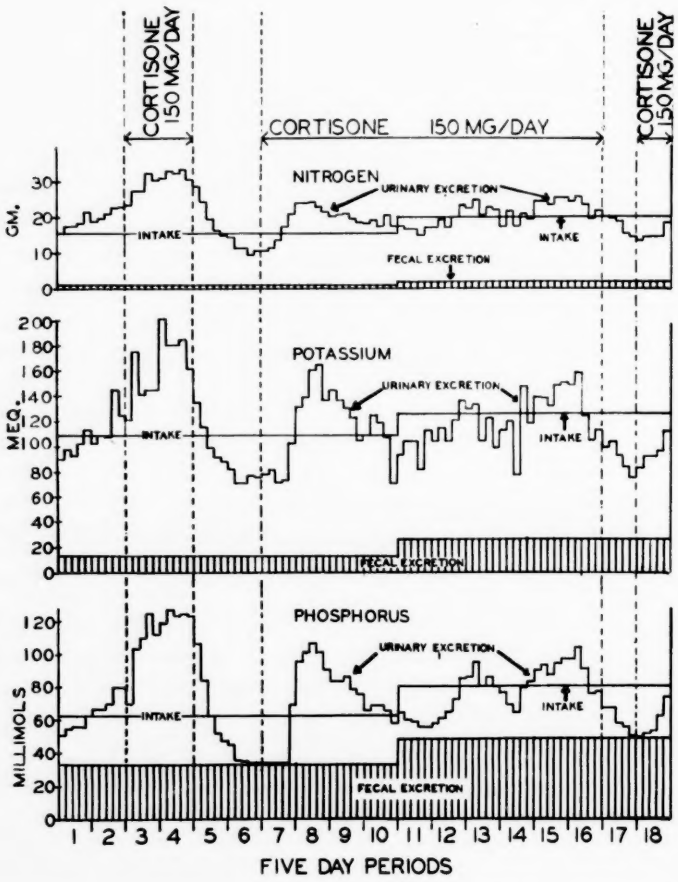

Fig. 1.—Case 1. Acute leukemia treated with cortisone showing exchanges of N, K, and P. The ordinates have been plotted so that one gram of N is equivalent to 2.7 meq. of K, and 2.15 millimols of P (Reifenstein and associates[38]). The phosphorus balance has not been corrected for the negative calcium balance, since the omission of this correction does not alter the interpretation of the figure.

Note: The negative balances of P and K, during the early phases of cortisone therapy, are greater than would be expected if the excess N, P, and K were derived from the catabolism of muscle. During the course of the balance study several transfusions were given as indicated below. No correction for the nitrogen excesses has been indicated in the above chart.

Period	*Nitrogen*
II	14.00 Gm.
III	11.70 Gm.
V	15.34 Gm.
VII	13.30 Gm.

(*1*), Vertically hatched area represents the fecal excretion. (*2*), Irregular line represents the urinary excretion. (*3*), Straight line represents the dietary intake (note that the diet was changed at the beginning of Period 11).

I. *Metabolic Data.*—Case 1 (K. N.), acute myeloblastic leukemia in whom cortisone appeared to induce a brief remission. The metabolic investigation revealed some striking changes. (Figs. 1 through 5.) A summary of these observations is as follows:

1. Negative nitrogen, potassium, and phosphorus balances were induced by cortisone (Fig. 1). However, the catabolic effect of the steroid gradually diminished during successive periods of therapy.

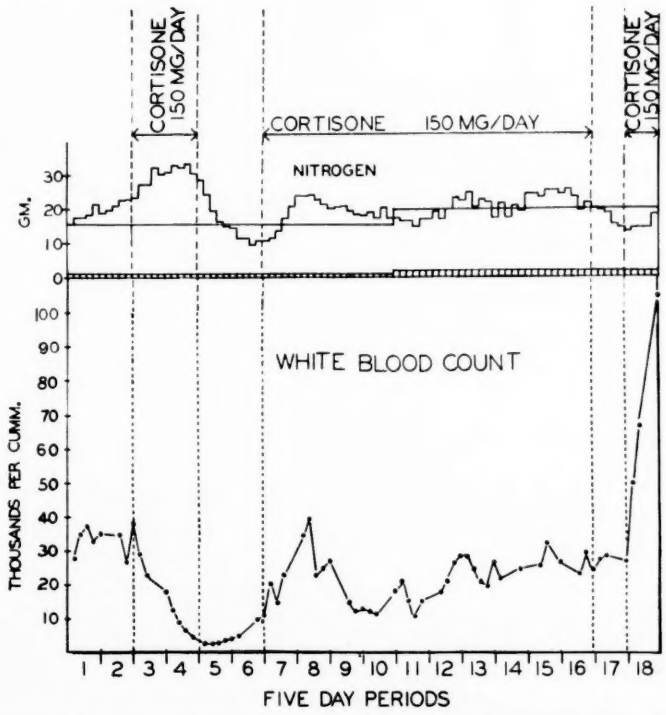

Fig. 2.—Case 1. Acute leukemia treated with cortisone, showing that the total white blood cell count was falling when the patient was in marked negative N balance, induced by cortisone.

2. The curves of the negative potassium and phosphorus balances were similar (Fig. 1). This suggests a common origin of these elements with respect to the tissue catabolized. In both cases the early loss was greater in proportion to nitrogen than could be accounted for if muscle protoplasm were the chief source of the catabolites.

3. During both courses of cortisone therapy there was a marked fall in the total white blood count. A rise in count followed the cessation of therapy in each instance. At the time that the white cell count was falling, the patient was in marked negative nitrogen, potassium, and phosphorus balance. Whether or not there is significant correlation between these findings cannot be ascertained (see Figs. 1 and 2).

4. The withdrawal of cortisone resulted in a rapid accumulation of phosphorus to the extent that the urinary excretion of phosphorus fell virtually to

zero during period 6 and the first three days of period 7. Also during the withdrawal period the serum phosphorus decreased (Fig. 4) from a pretreatment value of 5.0 mg. per cent to 1.9 mg. per cent, and a prompt return to 6.0 mg. per cent followed reinstitution of cortisone therapy. The N/P ratios calculated by the method suggested by Reifenstein and associates[28] did not follow any

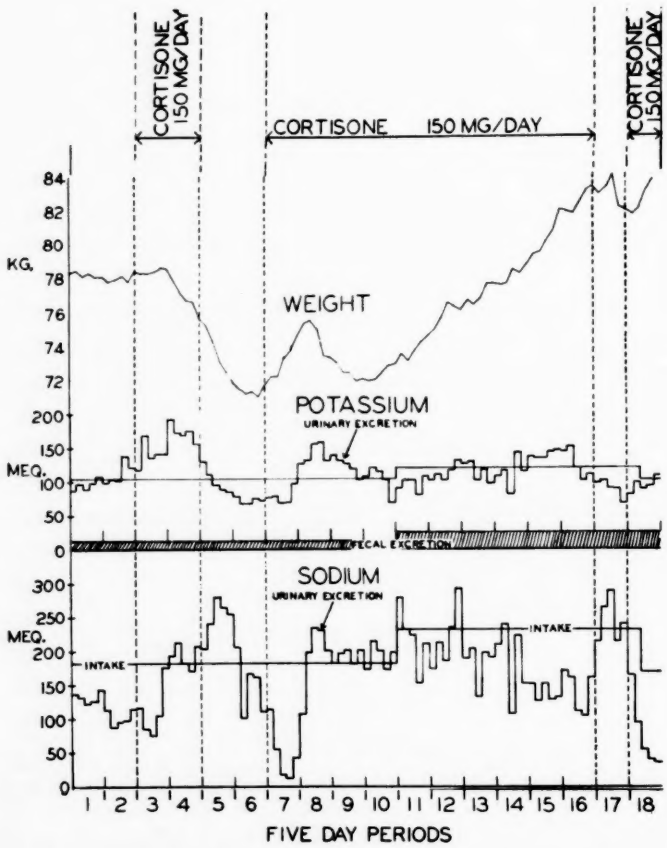

Fig 3.—Case 1. Acute leukemia treated with cotrisone, showing the Na and K exchanges and their relationship to the body weight.

consistent pattern, and were not those which one might expect if due chiefly to the anabolism or catabolism of muscle. During the early marked catabolic response apparently initiated by cortisone (periods 3 and 4), the ratio was 9.1, while later (periods 13 through 16) it had increased to 47. Ratios computed for periods of nitrogen and phosphorus retention also revealed great variability. In period VI, the second control period after the initial course of cortisone, N/P = 4.7, in periods XI and XII, following the increase in the diet, N/P = 2.9. The significance of these findings is difficult to evaluate. The variations in phosphorus exchange as observed in this individual were even more pronounced than those reported by Pearson and Eliel[10A] in their studies of leukemia and

other malignant disorders. To our knowledge, these extreme variations in phosphorus exchange have not been noted in other malignant disorders treated with cortisone.

5. The calcium and phosphorus balances are charted by periods in Fig. 5. It will be observed that there was a negative calcium balance throughout the period of study, that the fecal excretion of calcium almost equaled the dietary intake, and, further, that there was no apparent correlation between the calcium and phosphorus balances. Whether this means that this patient merely did not

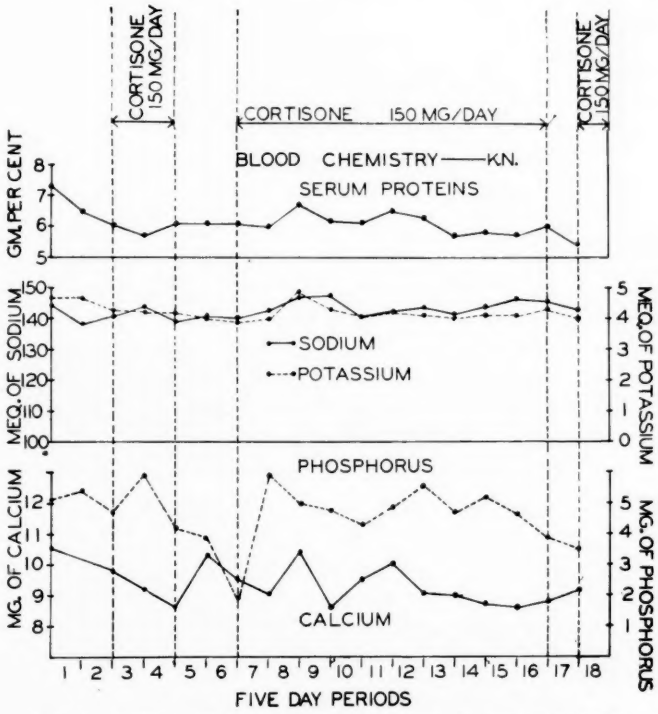

Fig. 4.—Case 1 (K.N.). Blood chemistry in acute leukemia.

absorb calcium from the gastrointestinal tract and consequently the urinary calcium represented mobilization of endogenous calcium stores, is a matter of speculation. In this case, it cannot be stated that cortisone materially affected the calcium balance.

6. There was a significant relationship of the body weight to the sodium balance. It will be noted in Fig. 3 that in the initial phases of the study (periods 1 through 3) the weight was nearly constant at 78 kg. Thereafter (periods 4 through 6) there was a sharp decline in weight from 78 kg. to 71 kg., probably related to the marked catabolic effect of cortisone as indicated by the negative nitrogen balance plus the sodium diuresis which resulted from cortisone withdrawal at the end of period 4. Cortisone therapy reinstituted at the beginning of period 7 caused an increase in weight from 71 kg. to 75.7 kg. This must have been related to sodium retention as the patient was in negative nitrogen balance.

Further continuation of cortisone at the same dosage and the same sodium intake was followed by a small sodium diuresis and consequent loss of weight (3.5 kg.). A change in the dietary intake of sodium from 182.7 meq. to 233.0 meq. per day resulted in a sustained weight gain, accumulation of obvious edema and prolonged positivity of sodium balance. Finally, near the end of the study, withdrawal of cortisone resulted in a small loss of weight and a small loss of sodium, even though the patient was nearly terminal.

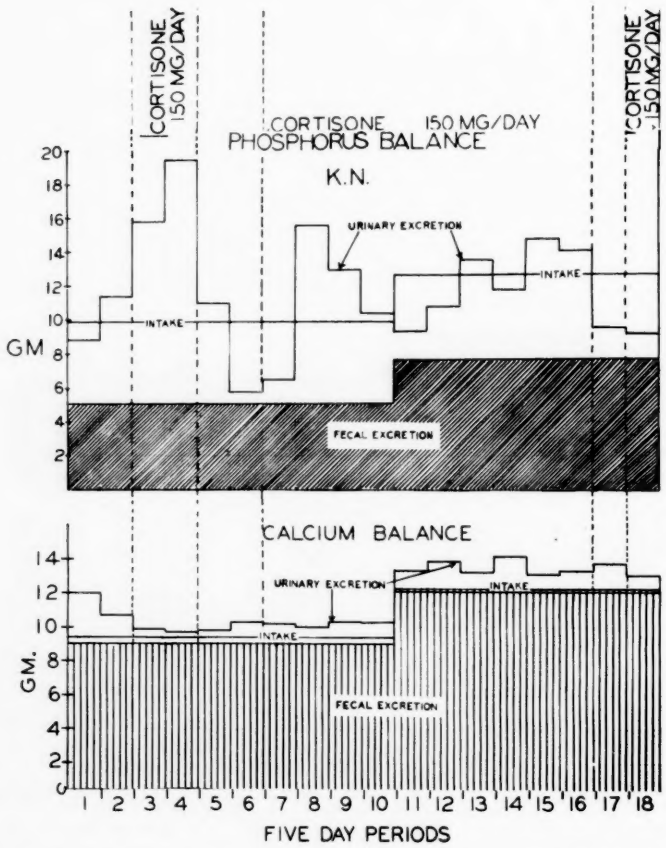

Fig. 5.—Case 1. Acute leukemia treated with cortisone, showing the Ca and P balances by five-day periods.

Improvement in this patient coincided with sharp negativity of the nitrogen, phosphorus, and potassium balances. When cortisone was withdrawn, there was a prompt reversal of these effects and an aggravation of the patient's disease. From the data obtained one might surmise that cortisone accomplishes its desired effect by making the necessary building blocks for cellular proliferation less available. This is not a new concept, and has been postulated previously from the results of animal experimental work.[3] However, the mechanisms of action of cortisone remain obscure.

TABLE I. SUMMARY OF CASES AND RESULTS OF TREATMENT

CASE NO.	PATIENT	AGE	SEX	TYPE OF LEUKEMIA	THERAPY			CLINICAL RESULTS*
					MATERIAL	DAILY DOSE MG.	NO. OF DAYS	
1	K.N.	23	M	Acute	Cortisone	150	80	Clinical and hematologic remission—approximately 1 month.
2	H.L.	38	M	Acute Myeloblastic	Cortisone	150	7	Clinical and hematologic remission—approximately 2 months.
					Cortisone	150	22	
3	A.S.	31	F	Acute Myeloblastic	Cortisone	100	15	Reduction in size of liver and spleen; otherwise not improved.
4	J.G.	5	F	Acute Lymphoblastic	Cortisone	75	15	Clinical and hematologic remission—one month.
5	G.W.	58	F	Acute Lymphoblastic	Cortisone	100	12	No significant effects.
6	D.B.	28	M	Acute Myeloblastic	Cortisone	200	17	Decrease in swelling of cervical lymph nodes; otherwise not improved.
7	W.H.	28	M	Subacute	Cortisone	150	9	No improvement.
8	A.B.	66	M	Subacute	ACTH	50	15	Healing of perirectal abscess; otherwise no benefit.
9	E.W.	36	M	Subacute Lymphocytic	ACTH	40	7	Excellent clinical and hematologic remission.
					ACTH	100	90	
10	G.M.	56	M	Chronic Lymphocytic	Cortisone	150	37	Temporary improvement.
					ACTH	40	30	

*All the patients have succumbed at the time of publication.

II. *Clinical Results* are summarized in Table I. Three of the ten cases treated with either cortisone or ACTH showed no improvement in their condition. Seven cases manifested some evidence of improvement such as cessation of hemorrhage and increase in the numbers of circulating blood platelets (Cases 1, 4, 5, 9, 10), involution of lymph nodes (Cases 1, 2, 6, 9, 10), shrinkage in size of spleen and liver (Cases 1, 2, 3, 9, 10), maintenance of hemoglobin and red cell values (Cases 1, 6, 10), decrease in white blood cell count (Cases 1, 2, 3, 9), tendency to increased maturation of leukocytes in the peripheral blood (Cases 4, 9), morphologic improvement in the bone marrow (Cases 4, 9), and reduction in fever and toxicity (Cases 1, 2, 4, 5, 9, 10). It must be stated, nevertheless, that in all except two cases, despite marked changes in other hematopoietic organs, definite morphologic evidence of the disease still existed in the bone marrow.

The immediate effects in certain individuals were so remarkable, however, that they merit further comment. In Case 2 (H.L.) the initial course of therapy resulted in an undoubted clinical and hematologic remission at a time when he appeared moribund. The rapid reduction in the size of the spleen and lymph nodes in the matter of a few hours was without precedent in our experience with other forms of therapy. Also of interest was the marked decrease in total leukocyte content which accompanied both courses of cortisone (Fig. 6). Notwithstanding, improvement was of a short duration and the beneficial effects of the second course of treatment, while quite definite, were not as pronounced as those of the first, and were shortly followed by clinical relapse while he was still receiving the steroid.

Case 10 (G.M.), a patient with chronic lymphocytic leukemia, had a peculiar "paradoxical" response to cortisone (Fig. 7). Although clinical improvement in this instance was obtained with cortisone administration, it is to be noted that the hematologic response was one of increased numbers of circulating lymphocytes at a time when the lymph nodes and spleen were decreasing in size. This illustrates the fact that the number of circulating lymphocytes may have little relationship to the quantity of lymphoid tissue.

The most notable results obtained in an adult were observed in Case 9 (E.W.) who had subacute leukemia, possibly of the lymphocytic type. He was given 100 mg. of ACTH daily for three months. Clinical and hematologic im-

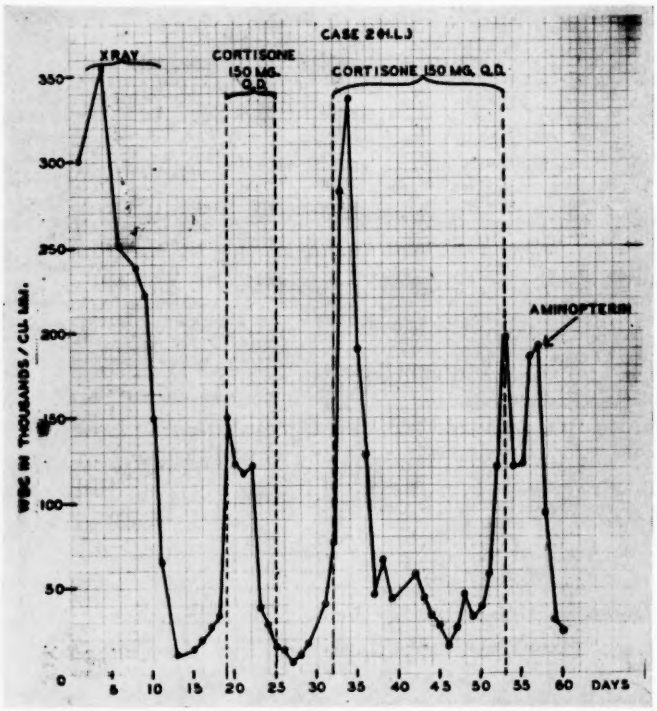

Fig. 6.—Case 2 (H. L.), showing the response of the white blood cells to cortisone administration in acute leukemia.

provement occurred after three weeks of therapy and at the present time (three months later) it is impossible to discern any departure from normal in the peripheral blood, although the bone marrow still contains a small number of immature leukocytes, probably early lymphocytes. An unfortunate accompaniment to the definite hematologic remission was the occurrence of a psychotic pattern of behavior (Fig. 8).

Undesirable effects of ACTH or Cortisone therapy in this group of patients were the production of a Cushing-like syndrome, glycosuria, hypertension, acne, bizarre mental aberrations (catatonic and manic states, marked mental depression, etc.—these on high doses), cerebral edema, peripheral edema and sodium

retention, hypopotassemia, adrenal cortical involution, and an Addison-like syndrome upon withdrawal. Lack of any benefit despite massive doses of ACTH or cortisone was noted a few times, also early relapse during therapy after an initial short period of improvement.

In general, it is the opinion of the authors, that, while these hormones may produce clinical remission in a few cases of leukemia, they are far from effective therapeutic agents. It appears to be difficult to avoid the undesirable side actions which have occurred all too frequently. To prevent a tendency toward excessive retention of sodium and development of edema it is suggested that patients be placed on a low sodium regime (probably not more than 800 mg.

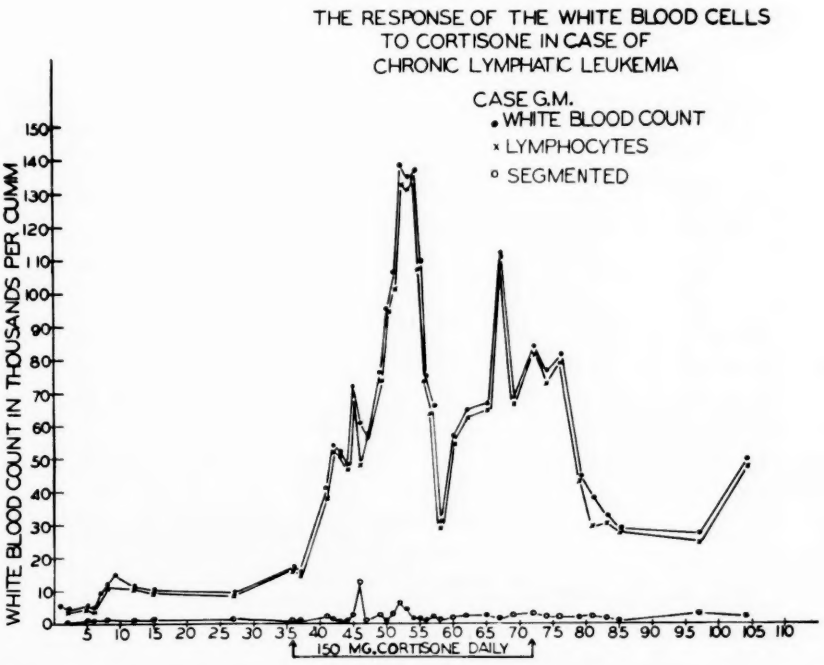
THE RESPONSE OF THE WHITE BLOOD CELLS
TO CORTISONE IN CASE OF
CHRONIC LYMPHATIC LEUKEMIA
CASE G.M.
• WHITE BLOOD COUNT
x LYMPHOCYTES
o SEGMENTED
WHITE BLOOD COUNT IN THOUSANDS PER CUMM
150 MG. CORTISONE DAILY

Fig. 7.—Case 10. Chronic lymphocytic leukemia, showing the rise in circulating lymphocytes which was coincident with the cortisone therapy.

sodium per day). The concentration of serum potassium should be followed at frequent intervals, especially when high dosages are employed, since hypopotassemia may be a complication. Prolonged use of a steroid, such as cortisone, can lead to involution of the adrenal cortex, as was observed at post mortem in Case 1. This complication may be difficult to circumvent, since withdrawal of the hormone leads to a relapse accompanied by intensification of all the symptoms and signs of the disease. It is possible that these involutionary changes could be obviated by the judicious use of both ACTH and cortisone, the former to maintain the adrenal cortex at full functional capacity, while the latter insures a high concentration of steroid in the tissues. The tendency to temporary failure of adrenal cortical function with the production of a syndrome re-

sembling Addison's disease can no doubt be lessened by gradual reduction in the size of the dose of the agent being employed rather than its abrupt withdrawal. The details of such a procedure have yet to be evaluated.

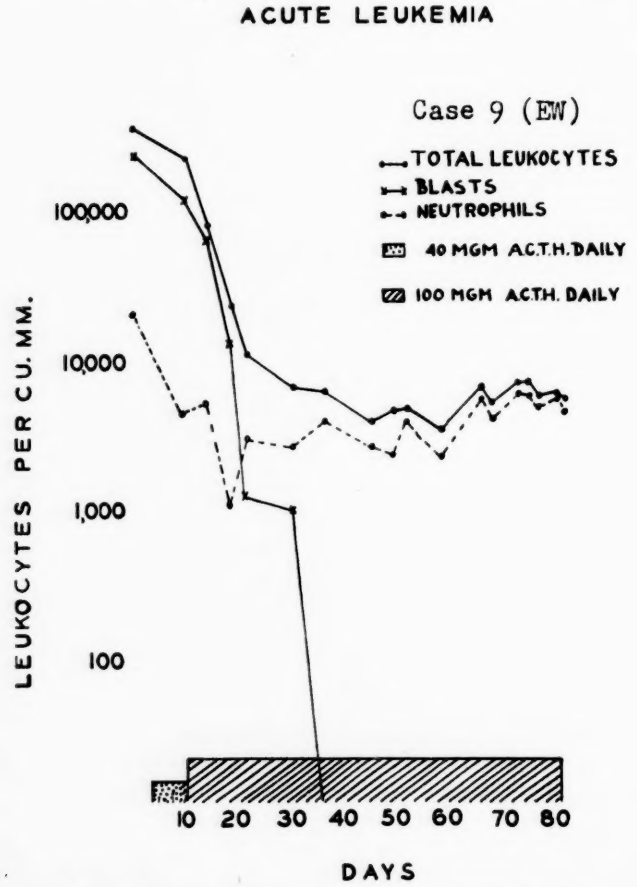

Fig. 8.—Case 9. Acute leukemia treated with large doses of ACTH, showing the change in the peripheral blood count and differential formula, coincident with this therapy.

CONCLUSIONS

Ten cases of leukemia of various types have been treated with ACTH or cortisone. Three of these cases showed no improvement from this form of treatment. Seven cases manifested some type of favorable response, but only five cases can be said to have had a remission in the course of the disease. Possible mechanisms of the remission are discussed in the light of metabolic data obtained in one case.

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ON THE DETERMINATION OF PACKED CELL VOLUME

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THE determination of packed cell volume* was made as early as 1892. In this country Capps[1] was probably the first investigator to use the procedure, centrifuging in the absence of an anticoagulant. Somewhat later Larrabie[2] introduced oxalic acid-sodium oxalate as the anticoagulant. The use of this mixture was based on the experiments of Grovitz. More stringent conditions were imparted to the procedure by Osgood,[3] who used a relatively large oxalated blood volume (4 ml.) and centrifuged at a speed of over 4,500 r.p.m. for five-minute periods until successive readings showed no change. The time consumed was from one-half to somewhat over one hour.

The present form of the method was introduced by Wintrobe.[4] Blood is first treated with dry potassium oxalate, 10 mg. for each 5 ml. blood. As a result there is a shrinkage in cell volume of 8.2 per cent. To compensate for this error the volume of packed cells is multiplied by a correction factor, 1.09. The hematocrit tube is filled to 100 mm. and centrifuged at 3,000 r.p.m. (centrifuge head, 9 cm. radius) until no further packing occurs. This usually takes from twenty to thirty minutes. The determinations are accurate to within 0.5 per cent and the probable error is much less than that of a chamber count.

Millar[5] introduced the concept of centrifugal force which has been generally overlooked or ignored. A second concept of fundamental importance is that of volume concentration of erythrocytes and its influence on the velocity of cell fall through a cellular suspension.[6]

Norris and Vagon[7] were impressed "with the value of the hemoglobin-erythrocyte-hematocrit ratio as an aid in management of certain battle injuries." Accordingly, they suggested the use of this relationship in the form of a chart to guard against errors when reporting laboratory results.

The determination of packed cell volume can be accomplished with less statistical error than certain other hematologic procedures, such as chamber and spectrophotometric erythrocyte count and hemoglobin concentration. The method being simple should yield acceptable results even when performed by relatively inexperienced laboratory trainees. Because of these facts, and since many individual determinations can be made simultaneously, this study was undertaken. The primary object was to correlate packed cell volume with erythrocyte count (chamber procedure as the standard method) so that the former, if accept-

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*Although the hematocrit and packed cell volume are terms commonly used synonymously, in this paper we adhere to the original differentiation, i.e., hematocrit is the graduated tube employed in the determination of packed cell volume.

able, could be substituted as the routine procedure. It is recognized that this is possible only when red-cell dimensions remain within normal limits. In cases where cells are abnormal, an accurate count can be made only by means of the counting chamber, for in this method a single red cell is a unit, irrespective of size.

METHODS

Wintrobe hematocrit tubes were used exclusively. Five were picked at random and calibrated with mercury. The average volume was 0.815 ml., with a range of 0.668 ml. to 0.977 ml.

All centrifuging was done in an International Centrifuge Size I, type SB. Each instrument was calibrated with the cover down, by using a revolution counter and stop watch, at varying speeds as indicated by the rheostat scale. Readings were made in triplicate. The attached tachometer was then calibrated, using the values obtained by the above instruments. Speed as r.p.m. by the two procedures was plotted against rheostat readings. The two methods agreed within from 1 to 3 per cent.

Five ml. of all blood samples were shaken with a dry mixture of ammonium and potassium oxalate contained in 0.5 ml. of a mixed solution (6 and 4 Gm. of the salts, respectively, per 500 milliliter).

Spencer counting chambers were used for erythrocyte counts, five squares being counted in the manner recommended. Twenty cubic millimeters of whole blood were pipetted into 8 ml. of Gowers solution, and the red cell concentration determined spectrophotometrically. The spectrophotometric analysis for hemoglobin was made by adding 20 cu. mm. of whole blood to 8 ml. 0.1 per cent sodium carbonate.

For the estimation of packed cell volume, hematocrit tubes were filled to 100 mm. and centrifuged at different speeds for varying periods of time. Individual variations will be described later.

STATISTICAL VARIATION OF THE METHODS

In order to evaluate the methods used in this study, a single blood sample was obtained and determinations made for erythrocyte count, hemoglobin, and packed cell volume by the methods previously described. A preliminary experiment had shown that, with the centrifugal instruments available, a speed of 2,500 r.p.m. and an effective radius of 15 cm., thirty minutes was sufficient to reach minimum values. This technique was used in determining packed cell volume (Table I). The fifteen trials represent triplicate estimations made by five technicians. In making the chamber counts fifteen pipettes and five counting chambers were used. All these values are in good agreement with those obtained by Ham.[8] The only exception is the chamber count which in this study has approximately one-half the coefficient of variation. A comparison of these values (C.V.) leads to the conclusion that the determination of packed cell volume can be accomplished with less variation than is obtainable with the chamber and spectrophotometric methods for erythrocyte count. Consequently, a red blood cell count based on the volume of packed cells will likewise show a smaller variation.

TABLE I. STATISTICAL ANALYSIS OF A SINGLE BLOOD SAMPLE

ERYTHROCYTE COUNT BY	UNIT	NO. OF TRIALS	MINIMUM VALUE	MAXIMUM VALUE	MEAN VALUE	2 S.D.	2 C.V. PER CENT
Chamber	10^6/cu. mm.	15	4.6	5.2	4.9	±0.38	±7.8
Spectrophotometer	10^6/cu. mm.	15	4.8	5.3	5.0	±0.28	±5.6
Packed Cell Volume*	10^6/cu. mm.	15	4.9	5.0	5.0	±0.16	±3.2
Hemoglobin, Spectrophotometric	Gm./100 ml.	15	13.6	14.8	14.1	±0.80	±5.6
Packed Cell Volume	mm.	15	43.0	44.0	43.3	±0.92	±2.3

*Erythrocyte concentration corresponding to packed cell volume, taken from Fig. 1.

RESULTS

Relation of Packed Cell Volume to Erythrocyte Chamber Count.—Inasmuch as the volume of packed cells in a given amount of blood is a function of erythrocyte concentration, there should be a linear relationship between the two. Accordingly, eight blood specimens were obtained, four female (type A) and four male (type O). Each sample was first centrifuged, and a portion or all of the plasma removed. The cells were suspended in the concentrated form or diluted either with varying quantities of physiologic saline solution or homologous plasma. The resulting concentrations ranged from 0.5 to 9.0 million cells per cubic millimeter. A chamber count (in triplicate) and a packed cell volume determination (in duplicate) was then made on each sample. The values, plotted, are shown in Fig. 1.

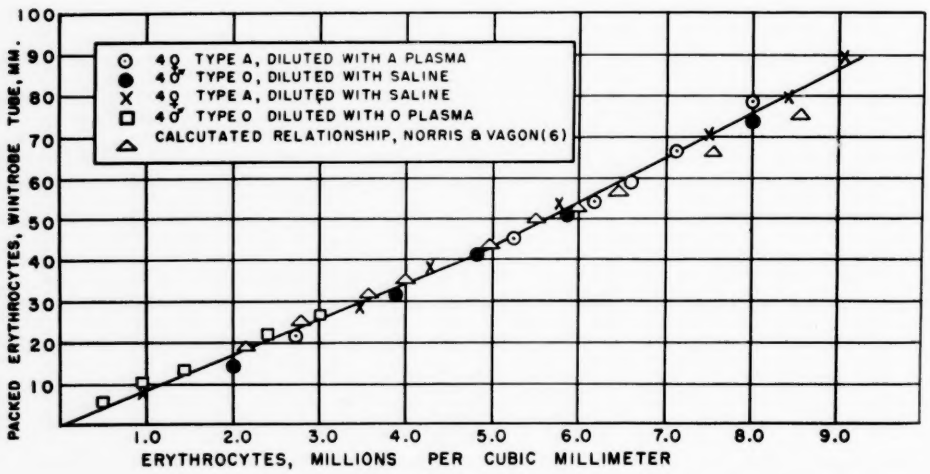

Fig. 1.—Relationship between packed erythrocytes and red cell chamber counts, experimentally determined by concentrating or diluting blood from normal individuals.

The first ten millimeters of packed cells are equivalent to 1.1 million erythrocytes per cubic millimeter. This value increases slightly per 10 mm. increment up to a packed cell volume of 43 mm., with an average per 10 mm. over this range of 1.12. At this point the curve breaks, and from 50 mm. to 80 mm. the apparent number of erythrocytes per 10 mm. gradually decreases to 1.06 million per cubic millimeter.

There are two possible reasons for this. The packed cell volume is too high (plasma is still retained in the interstitial spaces), or the erythrocyte count is too low. A centrifuge of extremely high speed was not available so that the possibility of a further reduction in cell volume could not be explored. Therefore the second of the two possibilities was studied. Accurate counting of red cells at high concentrations is made difficult owing to the masking of cells in the lower layers. To check this point, three blood samples were obtained with concentrations of 1.2, 4.6 and 5.4 million cells per cubic millimeter (Fig. 2).

Chamber counts on the samples were made in the following manner: for a single sample, six blood pipettes were used and each filled with a different quantity of blood. The volumes taken were equivalent to the 0.1, 0.3, 0.5, 0.6, 0.8 and 1.0 divisions on the pipette scale. A full complement of diluting fluid was then drawn into each pipette, and the sample thoroughly mixed. Chamber counts were made in the usual manner.

The effect of cell concentration is plainly seen. When red cells are not too numerous, equal counts can be obtained with any volume of blood from 0.1 to 1.0 divisions (Fig. 2, curve A). With increased erythrocyte concentrations, volumes over the 0.5 division tend to give results which are too low (Fig. 2, curves B and C). No attempt was made to determine exactly the point or, what is more probable, the area through which the concentration effect becomes markedly manifest.

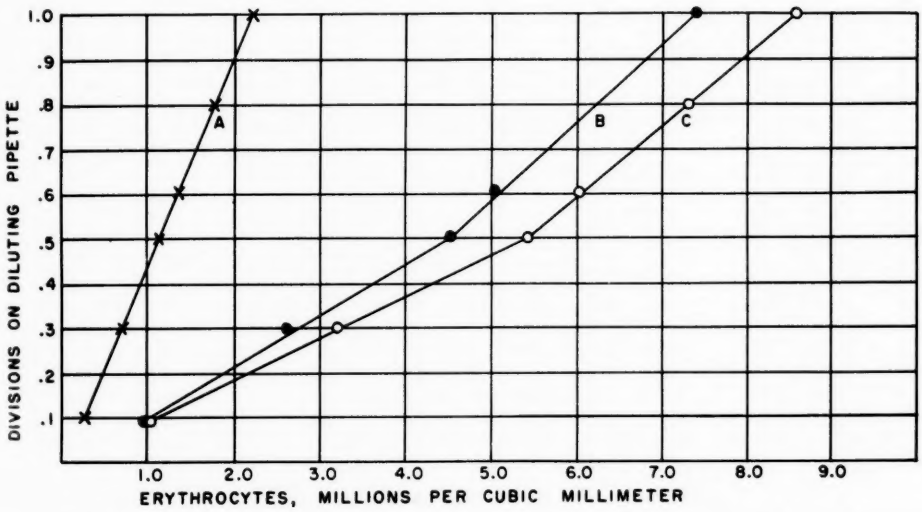

Fig. 2.—Effect of erythrocyte concentration on the accuracy of the chamber counting procedure using three bloods of varying red cell concentration, A 1.2, B 4.6, C 5.4 million cells per cubic millimeter.

The assumption has been made in this study that the erythrocytes in 0.1 division of a pipette can be accurately counted due to noninterference. Therefore multiples of this count (0.1 division) should be equal to the number of cells found in a volume equal to the multiple. For example, if in 0.1 division the count is 0.25 million, then in 0.2 division the count should be 0.5 million and in 0.5 division 1.25 millions. This would not be true if the number of cells had an interfering effect.

The values in Table II show that for a blood specimen of low cell concentration, the quantity of blood pipetted is of little moment. Variation is small, amounting to 0.03 to 0.08 million cells per cubic millimeter over the entire range. Whereas, in the two blood samples of higher cell concentration the erythrocyte concentration values up to the 0.5 division are within 0.2 million cells per cubic

millimeter, the count in a volume greater than the 0.5 division falls off considerably. The break in the curve (Fig. 1) can be explained in part by the low erythrocyte count when cell concentration is high.

TABLE II. ACCURACY OF ERYTHROCYTE COUNT IN RELATION TO TOTAL CELL CONCENTRATION BASED ON NUMBER OF CELLS IN 0.1 DIVISION OF DILUTING PIPETTE

A	B	C	D
DIVISIONS ON PIPETTE	ACTUAL CHAMBER COUNT AT THE INDICATED PIPETTE DIVISION 10^6/CU. MM.	ACTUAL CHAMBER COUNT AT 0.1 DIVISION × THE NUMBER OF DIVISIONS FILLED AS WHOLE NUMBERS 10^6/CU. MM.	DIFFERENCE COLUMN B − C 10^6/CU. MM.
	Curve A		
0.1	0.22	0.22	±0.00
0.3	0.74	0.66	+0.08
0.5	1.18	1.10	+0.08
0.6	1.40	1.32	+0.08
0.8	1.80	1.76	+0.04
1.0	2.23	2.20	+0.03
	Curve B		
0.1	0.95	0.95	±0.00
0.3	2.65	2.85	−0.20
0.5	4.55	4.75	−0.20
0.6	5.05	5.70	−0.65
0.8	6.00	7.60	−1.60
1.0	7.40	9.50	−2.10
	Curve C		
0.1	1.05	1.05	±0.00
0.3	3.15	3.15	±0.00
0.5	5.40	5.25	+0.15
0.6	6.05	6.30	−0.25
0.8	7.30	8.40	−1.10
1.0	8.55	10.50	−1.95

Norris and Vagon[8] utilized the hemoglobin-erythrocyte-hematocrit ratio as a check to guard against errors in reporting erythrocyte count and hematocrit values. Their chart was based on the normal values of 5.0 million red cells per cubic millimeter and on an average packed cell volume of 45 per cent. Variations in cell concentration, and consequently packed cell volume, were calculated by either increasing or decreasing the normal values by 1/40, 2/40, 3/40, etc. Some of their values for erythrocyte count and the corresponding volume of packed cells are plotted in Fig. 1 (triangles). The agreement, below a concentration of 5.0 million, with the experimental data presented here, is excellent. However, above 5.0 million their chart does not conform, and indicates a straight line relationship over the entire range of concentration. In the present study, if it be assumed that the packed cell volume is correct, and the chamber count above 5.0 million is too low, then a continuation of the curve (Fig. 1) as a straight line above the concentration of 5.0 million will give values which are more nearly correct. The calculated values[7] and those obtained by experimentation are then brought into agreement, and each hematocrit mm. equals 0.114 million erythrocytes.

TABLE III. RELATION OF R.P.M. AND TIME TO COMPLETENESS OF CELL PACKING

	R.P.M.	CENTRIFUGAL FORCE*	CENTRIFUGING TIME, MIN.						CENTRIFUGING TIME, MIN.					
			5	10	15	20	25	30	5	10	15	20	25	30
			R.B.C. 10^6/cu. mm.						Per cent of R.B.C. at 2,500 r.p.m. 30 min.					
Series 1 average 4 bloods	2,000	120	5.9	–	5.2	5.1	5.1	5.0	120	–	106	104	104	102
	2,500	187	5.2	–	4.9	4.9	–	4.9	106	–	100	100	100	100
Series 2 average 5 bloods each in duplicate	2,000	120	–	4.9	–	4.7	–	4.7	–	107	–	102	–	102
	2,500	187	–	4.7	4.6	4.6	–	4.6	–	101	100	100	–	100
Series 3 average 5 bloods	2,000	120	5.7	5.3	5.1	5.1	5.0	5.0	117	108	104	104	102	102
	2,500	187	5.2	5.1	4.9	4.9	–	4.9	106	104	100	100	100	100
Series 4 average 6 bloods	2,000	120	–	5.6	–	5.4	–	5.2	–	108	–	104	–	100
	2,500	187	–	5.4	–	5.2	–	5.2	–	104	–	100	–	100
	3,000	270	–	5.3	–	5.2	–	5.1	–	102	–	100	–	98
	3,500	367.5	–	5.2	–	5.2	–	5.2	–	100	–	100	–	100

*Circle of rotation 15 cm.

TABLE IV. RELATION OF R.P.M. AND TIME TO COMPLETENESS OF CELL PACKING AT LOW ERYTHROCYTE CONCENTRATION

R.P.M.	CENTRIFUGAL FORCE*	PATIENTS	CENTRIFUGING TIME, MIN.			CHAMBER COUNT
			10	20	30	
			10^6/cu. mm.			10^6/cu. mm.
2,000	120	Ni.	4.0	3.9	3.8	
		Mo.	2.9	2.9	2.8	2.9
		Ma.	3.9	3.9	3.8	4.0
		Br.	3.7	3.7	3.6	3.6
2,500	187	Ni.	3.9	3.9	3.8	
		Mo.	2.8	2.8	2.8	2.9
		Ma.	3.9	3.9	3.9	4.0
		Br.	3.7	3.6	3.6	3.6

*Circle of rotation (radius) 15 cm.

Minimum Conditions for the Determination of Packed Cell Volume.—The foregoing data indicate that in blood containing normal cells, there is a definite ratio between erythrocyte count and packed cell volume under the procedures described. The value of a method for clinical use depends in part on the accuracy and also on the rapidity with which it can be made. Accordingly a series of experiments were performed to determine the minimum speed and time of centrifuging which would give results of acceptable accuracy (Table III). Packed cell volume has been translated to red blood cell concentration using the curve in Fig. 1. A speed of 2,000 r.p.m. usually gives values ranging from 7 to 20 per cent higher than at 2,500 r.p.m., depending on the time of centrifugation. At 2,500 r.p.m. a constant volume of cells is obtained at the end of 15 to 20 minutes. Increasing centrifugal speeds to 3,000 and 3,500 r.p.m. has little or no effect in decreasing the time required for packing.

In the case of "bleeder" patients it is frequently desirable, and sometimes necessary, to have results in as short a period as possible. Four patients of this type were chosen and blood samples analyzed for packed cell volume and red cell count, Table IV. With a decreased cell concentration, 10 minutes seem sufficient to give minimum readings.

TABLE V. ERYTHROCYTE SIZE AND CORRELATION BETWEEN CELL COUNTS BY THREE METHODS

	ABNORMALITY	CHAMBER 10^6/CU. MM.	PACKED CELL VOLUME 10^6/CU. MM.	SPECTROPHOTOMETER 10^6/CU. MM.
1	Microcytic	3.8	3.0	2.6
2	Microcytic	2.5*	2.5	2.5
3	Microcytic	2.8*	3.0	2.8
4	Microcytic	4.6	4.0	3.5
5	Microcytic	5.7	5.1	4.4
6	Microcytic	5.6 (4.9)	5.0 (4.3)	4.4 (3.7)
7	Macrocytic	2.2	2.6	–
8	Macrocytic	2.0	3.0	2.5
9	Macrocytic	2.2	3.2	–
10	Macrocytic	3.9	4.8	–
11	Macrocytic	4.0 (2.9)	5.2 (3.8)	4.6 (3.6)
12	Polycythemia	7.8	7.3	7.8
13	Polycythemia	6.8	6.7	6.4
14	Polycythemia	7.5 (7.4)	7.0 (7.0)	6.8 (7.0)

*Values in Cases 2 and 3 not included in average.
Figures in parentheses average values within each type of abnormality.

The data indicate that maximum packing of cells in the normal concentration range, can be obtained in fifteen to twenty minutes at 2,500 r.p.m. However, thirty minutes are needed if the rate of spin is 2,000 r.p.m. From a practical point of view ten minutes are sufficient for centrifugation, provided a speed of 2,500 r.p.m. and a radius of 15 cm. or more is used. Under these conditions the greatest discrepancy will not exceed 2 to 4 per cent or 0.1 to 0.2 million erythrocytes per cubic millimeter of blood (Table III).

The Use of Packed Cell Volume (mm.) vs. Erythrocyte Count (millions per cubic millimeter).—So far we have been concerned with correlating packed cell volume with red cell count, merely because it is common practice to think in terms of millions of erythrocytes per cubic millimeter.

The average transition value found in this study is 1.12 million red cells per cubic millimeter, equivalent to 10 mm. of packed cells in the normal and subnormal range. However, translating packed cell volume to cell concentration should not be necessary once the clinical variation of packed cells is understood. The range for normal bloods in both sexes will be from 35 to 52 mm. of packed cells, equivalent to 4.0 to 5.7 million erythrocytes per cubic millimeter. In the anemias and "bleeders" the range will be from 10 to 30 mm., and in the polycythemias and the hemoconcentration cases, above 52 mm.

Effect of Abnormality in Size of Cells.—The point was made in the introduction that the use of packed cell volumes as a routine means of determining erythrocyte concentration will depend on the normalcy of cell dimensions. In the counting chamber, a single cell is a unit irrespective of size, whereas in an hematocrit tube, the amount of space occupied by a cell is a factor limiting technical accuracy. Any deviation in cell count by centrifugation due to an abnormality in size will depend on two factors: the degree of abnormality of each individual cell, and the total number of such cells present in a given volume of blood. Thus it is conceivable that a few cells greatly reduced or increased in size will have little effect, but a large number of cells, only slightly changed, may alter the packed volume considerably. The relative accuracy of the packed cell procedure as compared with chamber count in anemias will depend on the interplay of these two factors. Consequently it is conceivable that the two values might be comparable in some instances and at variance in others. To illustrate, a comparison was made by the three methods, using blood from twelve patients not having hematologic involvement. The average concentration figures, 5.1, 5.1, 4.9 million cells per cubic millimeter, were in satisfactory agreement for the chamber count, packed cell volume, and spectrophotometric procedures, respectively. Samples were then obtained from patients having red cell abnormalities. As was anticipated, the average values for the packed cell volume and spectrophotometric methods were too low when compared with a chamber count for microcytic cells and too high for macrocytic forms (Table V). In a few instances the three methods were in agreement. The cell count in three polycythemic bloods agreed on an average within 0.4 million cells or within approximately 5 per cent. These findings substantiate the fact that in cases of blood dyscrasias leading to microcytic or macrocytic

TABLE VI. HEMATOLOGIC CONSTANTS

CONSTANT	METHOD FOR ERYTHROCYTE COUNT	NO. CASES	MIN. VALUE	MAX. VALUE	AV. VALUE	NORMAL RANGE
			Cubic microns			
Mean corpuscular volume	Chamber	12	78	100	89	80-94
	Spectrophotometer	12	83	96	89	
	Packed cell volume	12	87	90	88	
			Micro micrograms			
Mean corpuscular hemoglobin*	Chamber	12	25	31	29	27-32
	Spectrophotometer	12	27	33	29	
	Packed cell volume	12	25	31	29	
Color index†	Chamber	12	0.84	1.02	0.96	0.97-1.08
	Spectrophotometer	12	0.90	1.10	0.96	
	Packed cell volume	12	0.84	1.02	0.95	

*Hemoglobin determined spectrophotometrically.

†Normal erythrocyte, 5.0 million per cubic millimeter, normal hemoglobin, 15.0 Gm. per 100 ml.

states, neither the packed cell volume nor the spectrophotometric method is reliable in determining erythrocyte count. Under these circumstances the chamber procedure should be used.

Hematologic Constants.—Because certain hematologic indices have diagnostic value, three of these were calculated, namely, mean corpuscular volume, mean corpuscular hemoglobin, and color index. The calculation involving packed cell volume is unusual in that this volume and its equivalent in erythrocytes (taken from Fig. 1) actually represents a single determination. The other two calculations were made from two determinations, i.e., red cell count (by the indicated method) and packed cell volume as used in the first calculation. Comparable values were obtained regardless of the method used in determining erythrocyte count (Table VI).

DISCUSSION

Centrifugal Force.—It is a common practice when describing methods for determining packed cell volume to state the conditions as r.p.m. for a definite time, i.e., 2,000 to 3,000 r.p.m. for thirty minutes. Actually we are concerned with something more fundamental than rate of speed, namely, centrifugal force. In a discussion of this force the following terms are included, mass of the object, angular velocity (r.p.m.) and radius (cm.). It is the latter value which, with few exceptions, seems to be so universally ignored.

The falling of red cells through a somewhat viscous fluid and the subsequent completeness of packing will be influenced by a number of factors. They are: (a) centrifugal force applied, speed, and effective radius, (b) duration of centrifugal spin, (c) volume concentration of cells in sample, (d) anticoagulants, and (e) secondary factors, such as bore of tube, state of aggregation, etc.

It should be understood that with sufficient spinning at any speed there is a minimum equilibrium volume, which is not necessarily the minimum packed cell volume.

Millar seems to have been the first to recognize that "the cell moves towards the bottom of the tube under the effect of its inertia manifested as 'centrif-

TABLE VII. CENTRIFUGAL FORCE AND PACKED CELL VOLUME

A*		B†		C‡		D§	
CENTRIFUGAL FORCE	VOLUME PER CENT PACKED CELLS (MM.)	C.F. FOR 30 MINUTES	VOLUME PER CENT PACKED CELLS (MM.)	C.F. FOR 10 MINUTES	VOLUME PER CENT PACKED CELLS, 45° C. (MM.)	C.F. FOR 10 MINUTES	VOLUME PER CENT PACKED CELLS, 10° C. (MM.)
1631	35.0 (4.00)§						
1176	35.2 (4.02)						
643	35.7 (4.05)			605	38.7 (4.40)	576	39.5 (4.5)
357	37.1 (4.25)	392	45.8 (5.18)				
		288	45.7 (5.18)				
189	39.0 (4.45)	200	46.3 (5.20)	225	39.7 (4.52)		
		128	47.5 (5.30)				

*Data from Millar.[5]

†Average of seven individual blood samples.

‡Average of three individual blood samples.

§Values in parentheses are erythrocyte counts in millions per cubic millimeter corresponding to packed cell volume according to the relationship established in Fig. 1.

ugal force,' which is proportional to the effective mass of the cell (allowing for the buoyancy of the medium), the radius of the circle in which it rotates, and the square of the angular velocity.'' He expressed the relation of these factors in the following equation*:

C.F. $= W^2 . r . 2/10^6$ (eq. 1) where W is angular velocity in r.p.m., r is average radius in cm. (measured from center of shaft to the midpoint of the packed cells in the hematocrit), and $2/10^6$ is a constant. In general, the mass (m) of single cells is considered to be similar and the term is dropped.

The relative effectiveness of speed and radius is demonstrated in the following calculations:

W r.p.m., 10^3	W^2 r.p.m., 10^6	r cm.	C.F. 10^6	Increase r.p.m. or r	Increase C.F.
2	4	16	64		
4	16	16	256	2× r.p.m.	4×
2	4	32	128	2× r	2×

Although angular velocity is twice as effective in increasing centrifugal force as the radius, this latter value cannot be ignored. Therefore, when stating the conditions under which packed cell volume is determined, the single value for centrifugal force and time of spinning is sufficient. From a stated value for centrifugal force one can easily calculate the r.p.m. necessary to satisfy the recommended centrifugal value by measuring the radius of the circle through which the tubes will travel, and substituting into the equation.

As the cells begin to pack, the radius becomes greater. In Millar's data the radius at 4,350 r.p.m. was 5.00 cm. and at 11,600 r.p.m. it was 6.06 cm. Measurements were taken to the center of the packed cells after equilibrium had been reached. However, an exact measurement of the effective radius for each sample is not clinically practical. The value for the radius was not so

*This is a modified form of the equation $F = \frac{mv^2}{r}$, where m is mass in grams, v is velocity in cm. per second and r is radius in centimeters, $\frac{v}{r}$ equals angular velocity and F is in dynes.

scrupulously measured in our study. The 15 cm. radius (referred to previously) is from the center axis to the midpoint of normal packed cell volume in the hematocrit. This means that for low counts the radius is actually greater than 15 cm. and the centrifugal force in proportion. The differences in radius between a packed cell volume of 44 mm. and 30 mm. is approximately 0.6 cm. in 15 cm. Using the radius values of 15 cm. and 15.6 cm. at 3,000 r.p.m., the centrifugal force values of 270 and 281 are found. This is a difference of approximately 4 per cent, which is not particularly significant.

The velocity of fall of a single spherical cell would follow Stokes' law, which includes such factors as viscosity and density of the liquid medium as well as the radius and density of the falling particle. When dealing with a suspension of cells, as in the case of blood, the rate of particle descent is, in addition, a function of volume concentration; i.e., the number of particles per unit volume, a value which increases as centrifugation proceeds. Some confirmatory evidence has been obtained to substantiate this. A comparison of Tables III and IV will show the influence of volume concentration as expressed in the time necessary for complete packing under the experimental conditions employed. The time for minimum values was less for bloods of abnormally low erythrocyte concentration than for those containing a normal quantity.

Effect of Centrifugal Force.—A possible source of error (perhaps less than 4 per cent) when using hematocrit tubes is the residue of plasma remaining in the interstitial spaces after centrifugation. Increasing quantities of plasma in these spaces enhances packed cell volume. Ponder has pointed out that when erythrocytes are more than 95 per cent packed, enormous forces are required to complete the process. With increasing centrifugal force Millar found an ever-decreasing cell volume (Table VII, A). However, at a centrifugal force of 1,631 (11,600 r.p.m., 6.06 cm. radius) packing had reached a maximum. The difference in results between the minimum and maximum centrifugal forces used, in terms of packed cell volume, was 4 mm., and for red cell count, 0.45 million per cubic millimeter, i.e. a difference of approximately 10.0 per cent. The data were derived from what Millar describes as a typical experiment using a single blood sample (10 mg. potassium oxalate per 10 ml.) and centrifuging ten minutes initially and then for successive five-minute periods to a constant volume.

Although the experiments in the present paper confirm the fact that packed volume approaches a finite volume as centrifugal force increases, the forces used are of a much smaller magnitude than those employed by Millar. (Tables III, IV, and VII, B.) There is, in addition, the difference in kind of anticoagulant and a possible discrepancy due to temperature. Millar's centrifugation was in air, while the present authors used an enclosed centrifuge. To investigate the possibility of temperature effects, a limited experiment was undertaken. Three blood samples were centrifuged in an International refrigerated centrifuge at 45° C. and 10° C. for ten minutes (Table VII, C and D). Although there is a suggestion that packing of cells at 10° C. is slower than at the higher temperature, the variation is not sufficient to account for the difference between Millar's results and those in this paper in terms of a temperature effect.

From a clinical laboratory point of view, if values can be duplicated under defined conditions, then absolute values, which are frequently difficult to obtain, especially in terms of time, are not always needed for adequate clinical interpretation. Therefore we accept relative values and the method employed becomes empirical. However, empiricism when kept at a minimum does not necessarily invalidate a convenient method.

Recommended Procedure for Determination of Packed Cell Volume.—(a) Five ml. of blood are placed in a bottle containing dried, 0.5 ml. of a combined solution (6 and 4 Gm. of ammonium and postassium oxalate, respectively, per 500 ml. of water) or two drops of depo heparin per 10 ml. blood and mixed with the anticoagulant. The hematocrit tube is then filled exactly to the 100 mark with a homogeneous sample.

(b) Centrifuge at a C.F. 187 or above for fifteen to twenty minutes, i.e., 2,500 r.p.m., radius 15 cm. Hematocrit mm. $\times$ 0.114 = R.B.C./cu. mm.

(c) A shorter time, ten minutes, may be used at C.F. 187 or above, with an error of approximately 5 per cent for normal red cell counts. When erythrocyte concentration is low, ten minutes at C.F. 120 (2,000 r.p.m., 15 cm.) or above, will give minimum values.

SUMMARY

The determination of packed cell volume can be adequately substituted for the erythrocyte chamber count as a routine clinical procedure. However, packed cell volume cannot be used satisfactorily to determine cell concentration when erythrocytes are either microcytic or macrocytic, except where such abnormalities are minimal. Under these circumstances a chamber count should be made.

The conditions of centrifugation used in the determination of packed cell volume should be expressed in terms of centrifugal force, thereby including the effect of radius as well as speed.

C.F. = r.p.m. $\times$ radius $\times$ $2/10^6$.

By substituting into the formula the effective radius (measured from center of centrifuge shaft to midpoint of packed cells, in cm.) and the desired C.F., the r.p.m. required to satisfy the equation can be calculated.

With normal bloods, C.F. 187 or greater, for fifteen to twenty minutes, is sufficient to obtain complete packing. A value within 5 per cent of the minimum can be obtained after 10 minutes. When the erythrocyte count is 2.0 to 4.0 million per cubic millimeter, C.F. 120 for ten minutes is sufficient.

Ten millimeters on the hematocrit scale are equivalent to 1.12 million erythrocytes per cubic millimeter up to approximately 50 millimeters. Above this mark the relationship is 1.06 million erythrocytes per cubic millimeter per 10 millimeters. However, if the curve, Fig. 1, is extrapolated above 40 mm. of packed cells, then each 10 mm. of the hematocrit tube will contain an average of 1.14 million erythrocytes (1.12 to 1.16) over the entire range, 0 to 90 mm.

Erythrocyte count as found by packed cell volume can be determined with less variation than by means of counting chamber or spectrophotometer procedures.

Hematologic constants can be adequately derived from packed cell volume expressed as concentration (millions per cubic millimeter).

Packed cell volume need not be translated into cell concentration. Familiarity with the former value and its clinical variations should be sufficient for clinical purposes.

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DETERMINATION OF TRAPPED PLASMA IN THE CENTRIFUGED ERYTHROCYTE VOLUME OF NORMAL HUMAN BLOOD WITH RADIOIODINATED (I^{131}) HUMAN SERUM ALBUMIN AND RADIOSODIUM (Na^{24})

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THE accuracy of the hematocrit reading as a measure of the relative corpuscular volume has become an increasingly important consideration in blood volume investigations, and in determinations of the erythrocyte concentrations of substances which are primarily extracellular.

The error of the hematocrit determination has been the subject of a number of studies.[1-17] Since cell compression apparently occurs only at extremely high speeds of rotation,[18] it has been recognized that this error is almost quantitatively due to plasma occluded in the centrifuged cell column. However, estimates of the difference between the hematocrit value and the true relative cell volume have ranged from −5 per cent to +9 per cent.[1-17]

Because of the wide variation in the reported values, the problem of trapped plasma has been reinvestigated here by the assay of radioactivity in whole blood, centrifuged erythrocytes, and supernatant plasma following the addition of Na^{24} or albumin labeled with I^{131}. Albumin I^{131} was found suitable for the purpose of the present study in view of the impermeability of the erythrocyte membrane to the large albumin molecule, the stability of the iodine-albumin bond,[19] and the natural occurrence of albumin in blood plasma. Although Na^{24} diffuses slowly into the erythrocytes,[20] it has provided additional evaluation of the upper limit of trapped plasma.

MATERIALS AND METHODS

The albumin I^{131} solutions were obtained from Abbott Laboratories. Dialyzed samples of these solutions were used in some instances.

Na^{24} was obtained from Brookhaven National Laboratories, supplied in aqueous solutions of $Na^{24}_2CO_3$. These were titrated with HCl to a pH of 7.4, evaporated to dryness, and reconstituted with isotonic saline solution to the desired concentration.

Wintrobe tubes were employed for the hematocrit determinations. Centrifugation was carried out for thirty minutes in a No. 2 International Centrifuge with a head assembly providing 3,000 r.p.m. by the tachometer reading when operated at the fiftieth step of the rheostat, and a corresponding maximum relative force of 2,080 g resulting at the tip.

Packed erythrocytes were obtained from centrifuged hematocrit tubes cut directly below the lower level of the buffy coat after most of the supernatant plasma had been

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removed. Since the pipetting of packed cells is not a standard procedure, Ostwald volumetric pipettes of 1.00 ml. capacity were calibrated for this purpose. By assay of radioactivity in one milliliter samples of plasma and of packed erythrocytes, obtained from blood specimens to which albumin I^{131} had been added, and in the washings of the residue in each pipette, it was found that an average excess residue of (5.0 ± 0.8) per cent of the volume of packed cells delivered remained in these pipettes.

Specimens to which albumin I^{131} had been added were assayed for radioactivity in one milliliter samples, diluted to 100 ml. with water in Marinelli beakers, placed around an RCL bismuth-walled gamma counter in a position of optimum geometric relationship to the counter tube. The sensitivity of this arrangement was 4,450 counts per minute per microcurie of I^{131}, and its background about 28 counts per minute.

Similarly, Na^{24} treated samples were assayed using a tetramethyl lead-filled, silver-walled glass gamma counter. The sensitivity of this arrangement was 6,000 counts per minute per microcurie of Na^{24}, and its background about 18 counts per minute.

Each counter tube was shielded by a 1-inch thick vertical lead housing, and attached to a scaling unit.

The time allowed for background and sample counting was such that their combined statistical variations resulted in a probable error of ± 1 per cent in the counting rates of packed erythrocytes, and a lesser error in the counting rates of blood and plasma. The counting rates obtained for packed cells were multiplied by a factor of 1.05 to correct for the determinate error of pipetting.

Appropriate corrections for physical decay of the isotopes were made where indicated.

Procedure.—Approximately 0.05 to 0.1 ml. of solution, containing about 10 μc of albumin I^{131} or 3 μc of Na^{24}, was added to 10 ml. of heparinized normal human blood in a test tube, and the contents mixed thoroughly by gentle inversion. Five hematocrit tubes were filled with the treated blood, allowed to stand for ten minutes, and centrifuged for thirty minutes at 3,000 r.p.m. The average hematocrit reading was obtained at room temperature. One milliliter portions of packed erythrocytes, whole blood, and plasma were individually assayed for radioactivity.

Calculations.—The values obtained by the direct method of determination with albumin I^{131} were calculated as follows:

$$V_{tp} = \frac{I_s^{131}}{I_p^{131}} \qquad (1),$$

where

V_{tp} = trapped plasma volume per milliliter centrifuged erythrocyte sediment,
I_s^{131} = I^{131} counts per minute per milliliter centrifuged erythrocyte sediment,
I_p^{131} = I^{131} counts per minute per milliliter supernatant plasma.

The values obtained by direct determination with Na^{24} were similarly calculated.

The indirect method of determination with albumin I^{131} involved the following calculations:

$$V_p = \frac{I_b^{131}}{I_p^{131}} \qquad (2),$$

$$V_c = 1 - V_p \qquad (3),$$

$$D = \frac{V_o - V_c}{V_o} \qquad (4),$$

where

V_p = plasma volume per milliliter whole blood,
I_b^{131} = I^{131} counts per minute per milliliter whole blood,
I_p^{131} = I^{131} counts per minute per milliliter supernatant plasma.

V_c = corpuscular volume per milliliter whole blood (calculated relative corpuscular volume value),

D = relative difference between the observed and calculated relative corpuscular volume values,

V_o = hematocrit readiing, including buffy coat (observed relative corpuscular volume value).

RESULTS

The results of experiments utilizing nondialyzed albumin I^{131} are shown in Table I. The mean value for trapped plasma thus obtained was of the order of 3 per cent.

TABLE I. DETERMINATION OF TRAPPED PLASMA WITH NONDIALYZED ALBUMIN I^{131}

SPEC. NO.	HEMATOCRIT* (PER CENT)	I_x^{131} COUNTS PER MIN. PER ML.	I_p^{131} COUNTS PER MIN. PER ML.	V_{tp} (PER CENT)
1	41.4	224	7848	2.86
2	51.5	205	6798	3.02
3	44.8	215	8070	2.66
4	38.6	205	7020	2.92
5	47.8	220	7780	2.83
6	50.7	251	8432	2.98
7	42.7	250	8388	2.98
8	48.0	295	8390	3.52
9	40.3	215	6126	3.52
10	35.6	207	6414	3.23
11	45.1	227	6664	3.41
12	40.0	190	6910	2.75
13	45.0	223	7630	2.92
14	45.6	216	7824	2.76
15	39.5	222	6670	3.32

The arithmetic mean value and probable error of $V_{tp} = (3.05 \pm 0.20)$ per cent.
*Hematocrit reading, excluding buffy coat.

Since the accuracy of the determination of trapped plasma is dependent on the requirement that the distribution of I^{131} in the blood be completely extracellular, the extent of protein-free activity in the albumin I^{131} solutions was investigated. It was found that an average of 3 per cent of the activity could be separated by dialysis in twenty-four hours, presumably as protein-free I^{131}. About 70 per cent of the total dialyzable activity was recovered during the first hour. It has been shown, in vitro[21] and in vivo,[22] that when I^{131} is incorporated into whole blood as KI, approximately 40 per cent of the activity of the blood is found in the erythrocytes when the hematocrit value is about 50 per cent. Calculated on this basis, approximately 1.2 per cent of the added activity of nondialyzed albumin I^{131} solutions may have been available to the erythrocytes. Consequently, correction for intracellular activity would bring the mean V_{tp} value to 2 per cent. This was corroborated experimentally, as shown in Table II, by using nondialyzed and dialyzed samples of albumin I^{131} in duplicate determinations. The $V_{tp}d$ value thus obtained, 2.13 per cent, provides a new limit to the maximum amount of trapped plasma present in the centrifuged erythrocyte volume.

In order to obtain some appreciation of the magnitude of the error of the hematocrit determination under varying conditions of centrifugation, the

TABLE II. COMPARATIVE DETERMINATIONS OF TRAPPED PLASMA WITH NONDIALYZED (N), AND DIALYZED (D) SOLUTIONS OF ALBUMIN IN I^{131}

SPEC. NO.	HEMATOCRIT (PER CENT)	I_s^{131} COUNTS PER MIN. PER ML.	I_p^{131} COUNTS PER MIN. PER ML.	V_{tp} (PER CENT)	DIALYZABLE I^{131} (PER CENT)
1 n	40.8	184	6256	2.94	2.5
d		156	7440	2.10	
2 n	41.5	160	5856	2.74	2.1
d		162	8760	1.85	
3 n	51.0	318	8826	3.60	4.4
d		186	7818	2.38	
4 n	47.5	198	6675	2.96	2.7
d		150	6819	2.20	
5 n	46.2	189	6545	2.89	2.5
d		185	8640	2.14	

The arithmetic mean value and probable error of $V_{tp}n = (3.03 \pm 0.22)$ per cent, and of $V_{tp}^d = (2.13 \pm 0.28)$ per cent.

TABLE III. COMPARATIVE DETERMINATIONS OF TRAPPED PLASMA WITH DIALYZED ALBUMIN I^{131} AT VARYING RATES OF CENTRIFUGATION

SPEC. NO.	HEMATOCRIT (PER CENT)	RATE R.P.M.	FORCE (× GRAVITY)	I_s^{131} COUNTS PER MIN. PER ML.	I_p^{131} COUNTS PER MIN. PER ML.	V_{tp} (PER CENT)
1	43.4	1,460	500	582	6,804	8.55
	41.9	2,140	1,000	312	6,804	4.59
2	47.7	2,600	1,500	180	6,819	2.64
	47.5	3,000	2,080	150	6,819	2.20

plasma residue in the packed red-cell column was determined in blood samples spun to constant volume at different rates, as shown in Table III. The results obtained suggest that centrifugation for thirty minutes at a force of about 2,000 g represents the optimum choice in the hematocrit determination, since the corresponding error is comparatively small, and appreciably larger forces are not readily attained with ordinary laboratory centrifuges. Also, there is no apparent indication that an increase in the force applied, within the noted range, compresses the cells, as borne out by the fact that the relative difference between the hematocrit readings of each blood specimen is in good agreement with the corresponding difference in the V_{tp} values observed.

In order to test further the possibility that fluid expression from the cells may occur, the albumin I^{131} concentration in supernatant plasma from blood allowed to settle without centrifugation was compared with that of the plasma from blood spun at forces of 2,000 g. Table IV gives the data on three experiments. No significant dilution of plasma activity occurred following cen-

TABLE IV. COUNTING RATES OF PLASMA SAMPLES FROM SEDIMENTED (S), AND CENTRIFUGED (C) BLOOD

SPEC. NO.	I_p^{131}	DIFFERENCE (PER CENT)
1 (s)	7848	0.0
1 (c)	7848	
2 (s)	6730	–1.0
2 (c)	6798	
3 (s)	8162	1.1
3 (c)	8070	

TABLE V. DETERMINATION OF TRAPPED PLASMA WITH Na^{24}

SPEC. NO.	HEMATOCRIT (PER CENT)	Na_s^{24} COUNTS PER MIN. PER ML.	Na_p^{24} COUNTS PER MIN. PER ML.	V_{tp} (PER CENT)
1	42.6	108	2972	3.66
2	50.7	136	3251	4.18
3	39.8	117	3142	3.73
4	35.6	193	4190	4.61
5	44.3	85	2714	3.17
6	40.0	133	3612	3.68
7	45.6	150	3430	4.38
8	38.2	114	3035	3.76

The arithmetic mean value and probable error of $V_{tp} = (3.90 \pm 0.31)$ per cent.

trifugation, demonstrating that the amount of fluid expressed from the cells, if any, is less than that neeeded to compensate for the amount of trapped plasma, and that the observed hematocrit value is not less than the true value.

The results obtained in Table V, by using Na^{24} for the evaluation of trapped plasma, have given a mean value of 3.90 per cent, which is almost twice as great as the $V_{tp}d$ value obtained from Table II. This suggests that nearly 2 per cent of the Na^{24} added to the blood specimen diffuses into the erythrocytes within the functional period of the experiment, i.e., about one-half hour. It was observed that at the end of three to four hours as much as 10 per cent of the sodium was in the cells.

The wide range of values which attend the indirect evaluation of the error of the hematocrit determination, indicated in Table VI, may be explained on the basis of the statistical variations involved and the manner in which they enter into the calculations. The calculated probable error of V_o is of the order of ±1 per cent, and the variations involved in pipetting and counting of blood and plasma samples result in an estimated probable error of ±2 per cent of V_c. However, the relative difference between these two quantities is appreciably small, theoretically of the order of trapped plasma, and, consequently, their combined variations inflict serious uncertainty in the values of D. Considering, as an illustrative example, a V_o value of (45.0 ± 0.5) per cent and a V_c value of (43.7 ± 1.0) per cent, then, from the mathematical treatment of these data, the following result is obtained for their relative difference and the attached probable error:

$$\frac{(45.0 \pm 0.5) - (43.7 \pm 1.0)}{45.0 \pm 0.5} = \frac{1.3 \pm \sqrt{0.5^2 + 1.0^2}}{45.0 \pm 0.5} = \frac{1.3 \pm 1.1}{45.0 \pm 0.5} =$$

$$0.029 \pm 0.029 \sqrt{0.85^2 + 0.01^2} = 0.029 \pm 0.025.$$

This result indicates that within 50 per cent of the cases one would expect D values in the range of 0.4 to 5.4 per cent, and that within 95 per cent of the cases one would expect values in the range of −4.4 to +10.2 per cent. It is clear, therefore, that the indirect method of evaluation of the error of the hematocrit determination does not provide an appropriate means of investigation.

TABLE VI. RELATIVE DIFFERENCE BETWEEN THE RELATIVE CELL VOLUME VALUES INDEPENDENTLY DETERMINED BY CENTRIFUGATION AND BY THE USE OF NONDIALYZED ALBUMIN I^{131}

SPEC. NO.	HEMATOCRIT (PER CENT)	V_c (PER CENT)	I_b^{131} COUNTS PER MIN. PER ML.	I_p^{131} COUNTS PER MIN. PER ML.	V_p (PER CENT)	V_c (PER CENT)	D (PER CENT)
1	41.4	41.9	4623	7848	58.9	41.1	1.9
2	51.1	51.4	3409	6798	50.1	49.9	2.8
3	44.8	45.3	4772	8070	59.1	40.9	9.7
4	38.6	39.1	4408	7020	62.8	37.2	4.9
5	47.8	48.3	3963	7780	50.9	49.1	−1.7
6	50.7	51.2	4248	8432	50.4	49.6	3.1
7	42.7	43.2	4856	8388	57.9	42.1	2.6
8	48.0	48.4	4202	8390	50.1	49.9	−3.1
9	40.3	40.7	3736	6126	61.0	39.0	4.2
10	35.6	35.9	4285	6414	66.8	33.2	7.5
11	45.1	45.6	3748	6664	56.8	43.2	5.3
12	40.0	40.5	4340	6910	62.8	37.2	8.2
13	45.0	45.3	4398	7630	57.6	42.4	6.4
14	45.6	46.0	4183	7824	53.5	46.5	−1.1
15	39.5	40.0	4065	6670	60.9	39.1	2.3

The arithmetic mean value and probable error of D = (3.5 ± 2.5) per cent.

DISCUSSION

The observation has been made that the hematocrit reading is significantly reduced when the speed of centrifugation is appreciably increased.[23] This has been critically appraised from the consideration of the possibility that extremely large forces compress the cells.[6] However, it has been calculated that the force required to produce complete separation of cells and plasma would also be sufficient to produce cellular disintegration.[24] Thus, the problem remains that it is virtually impossible to determine the relative corpuscular volume accurately by centrifugation.

Numerous investigators have sought an index relative to the completeness of cell packing effected by centrifugation, by a variety of methods which may be classified as follows:

1. The indirect methods are based on comparisons of the relative cell volume values obtained independently by centrifugation and by other means, such as: (a) the dye dilution method in which a colored substance, to which the erythrocyte is supposedly impermeable, such as the subject's own hemoglobin,[1, 6] or a dye such as vital red,[2-4] or Evans blue,[7, 11, 13-17] is added in known concentration to a volume of blood and to a volume of plasma from the same blood specimen; then by colorimetric assay of the dyed plasma in the two samples, the volume concentration of the cells in the sample of blood can be calculated; (b) a method based on the empirical observation that the true relative cell volume may be calculated from the observed rate of cell packing on extended centrifugation[5]; (c) the cell opacity method in which the relative cell volume is calculated from measurements of light transmission through a column of blood.[12]

2. The direct methods are based on determinations which permit calculation of the volume of plasma occluded in the interstices between the packed cells, such as: (a) determinations of the protein,[10, 14-16] electrolyte,[9] or dye[10, 11, 13-16] content of the supernatant plasma and washings of the plasma

TABLE VII. SUMMARY OF THE LITERATURE

AUTHOR	METHODS	RESULTS
Stewart[1]	Indirect, hemoglobinometry	Obtained higher relative plasma volumes by this method than by centrifugation
Keith and associates[2]	Indirect, dye* dilution	Obtained differences† of 0, 0.5 and 3.5 per cent
Hooper and coworkers[3]	Indirect, dye* dilution; used animal blood and different rates of centrifugation	Obtained differences of −1.8 and +4.2 per cent
Smith[4]	Indirect, dye* dilution; used animal blood	Obtained differences of −5, −3, −2, and +2.5 per cent
Hirota[5]	Indirect, extended centrifugation	Obtained differences in the range of 8 to 10 per cent for human blood spun at about 2,000 r.p.m. for thirty minutes, and lower values by prolonged centrifugation
Ponder and Saslow[6]	Indirect, hemoglobinometry; varied time of centrifugation	Obtained differences of −5, 0, +1, and +2.5 per cent
Wintrobe[7]	Indirect, dye dilution	Obtained about the same relative cell-volume values by this method as by centrifugation
Fricke and Curtis[8]	Direct, conductivity measurements	Observed that the cell residue contains from 2 to 5 per cent of the supernatant plasma.
Oberst[9]	Direct, sodium determinations	Found that 3 per cent of the supernatant plasma sodium remains in the cell residue
Crabtree and Maizels[10]	Direct, hemoglobin and protein determinations	Found that 5.5 per cent of the packed cell volume is trapped plasma
Gregersen and Schiro[11]	Direct and indirect, dye determinations	Obtained direct and indirect values of 3.9 and 4.2 per cent, respectively, for human blood
Shohl[12]	Indirect, cell opacity	Found relative cell-volume values by this method which agree within 2 to 3 per cent with the values obtained by centrifugation
Shohl and Hunter[13]	Direct and indirect, dye determinations; used strong dye solutions	Obtained direct and indirect values of 2 and 4.4 per cent, respectively
Chapin and Ross[14]	Direct and indirect, protein determinations and several variations of the dye method	Found that 8.5 per cent of the relative cell volume determined by centrifugation is trapped plasma
Maizels[15]	Direct and indirect, protein and dye determinations; used concentrated dye solutions	Found 2.25 per cent trapped plasma with buffy coat removed and 2.7 per cent with buffy coat retained: found similar indirect values
Mayerson and associates[16]	Similar methods as used by Chapin and Ross	Obtained same values as Chapin and Ross
Barnes and coworkers[17]	Indirect, micro-dye method; centrifugation at 1,500 g for thirty minutes	Obtained a difference of 5 per cent, and corroborated the indirect values obtained by Shohl and Hunter and by Gregersen and Schiro

*Dyes from the vital red series were used in these determinations; Evans blue was used in the rest of the dye determinations.

†This is the per cent difference between the hematocrit and dye values of the relative cell volume; negative values indicate that the dye value was higher than the hematocrit value.

residue in the centrifuged cell column; (b) determinations based on the principle that since erythrocytes are presumably nonconductors of electricity, the plasma content of the centrifuged cell volume may be evaluated from its conductivity.[8]

A summary of published studies is shown in Table VII. The results have been inconsistent and the reported values have ranged from −5 per cent to +9 per cent.

The variations encountered in the results of some of these investigations, by similar methods, may be attributable in part to the variable use of subjects, the preliminary treatment of the blood, the size of the hematocrit tube, and, to a larger extent, the time of centrifugation, the force applied, and the exclusion or inclusion of the buffy coat.

In view of the fact that the indirect methods are subject to large error, they do not afford satisfactory evaluation of the small amount of trapped plasma present in the centrifuged corpuscular volume.

In the direct methods of investigation the technical difficulties encountered in the procedure have been regarded as the principal source of error. Some of these have been eliminated in the present study by sacrificing the hematocrit tubes to avoid mixing of cells and plasma. In addition, the necessity of extracting the plasma residue by washing the column of packed cells has been obviated, since the technique employed permitted direct assay of the packed cells.

The results obtained here agree well with the lowest positive results obtained by other investigators, and do not support the concept that an appreciable amount of plasma remains in the packed erythrocytes under the specified conditions of centrifugation. The value of 8.5 per cent obtained by Chapin and Ross[14] has been widely used, and the relative cell-volume values thus calculated from the hematocrit value have been applied to theoretically important considerations, particularly in regard to the average body hematocrit, often leading to contradictory opinions.[16] It appears, from the experimental data reported here, that corrections for trapped plasma in excess of 2 per cent are not justified when forces of 2,000 g or more are used in centrifugation.

SUMMARY AND CONCLUSIONS

1. The error of the hematocrit determination, due to incomplete cell packing under specified conditions of centrifugation, has been evaluated by methods which involve the addition of radiosodium or radioiodinated human serum albumin to whole blood.

2. Direct estimates of the plasma residue in the centrifuged cell column, obtained by the assay of radioactivity in packed erythrocytes and in the supernatant plasma, yielded the following mean values and attached probable error:

a. with Na^{24}	(3.90 ± 0.31) per cent
b. with nondialyzed albumin I^{131}	(3.05 ± 0.20) per cent
c. with dialyzed albumin I^{131}	(2.13 ± 0.28) per cent

The values obtained with albumin I^{131} agree well with each other when corrections are made for the relative fraction of dialyzable I^{131} available to the erythrocytes. The somewhat higher values obtained with Na^{24} are explainable on the basis of the entrance of this cation into the red cells.

3. The error of the hematocrit determination has also been indirectly evaluated in terms of the relative difference between the relative cell volume values obtained independently by centrifugation and by the assay of radioactivity in whole blood and in plasma. The results thus obtained have given very variable values. These variations, inherent in the manner of estimation, have been analyzed and shown to impose serious limitations on the method.

4. The error of the hematocrit reading, due to incomplete cell packing, is not in excess of 2 per cent for normal human blood spun at 2,080 g for thirty minutes in Wintrobe hematocrit tubes.

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THE EFFECT OF ACTH, CORTISONE, AND OPERATIVE STRESS UPON BLOOD CHOLESTEROL LEVELS*

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Among the numerous metabolic changes which accompany the action of exogenous or endogenous adrenal cortical hormones, those of serum cholesterol have been mentioned frequently. With two exceptions[1, 2] reported studies have included a paucity of data, and there appears to be considerable variance of opinion regarding the type, degree, and causes of the observed changes. Conn, Vogel, Louis and Fajans[1] demonstrated a marked depression of serum cholesterol during the administration of ACTH to normal persons as opposed to lack of such response in patients with Addison's disease. They noted a slight and poorly maintained depression of serum cholesterol during treatment with cortisone. The most marked ACTH induced depression of cholesterol occurred in a patient with Cushing's syndrome, a finding also noted by Lewis and Wilkins.[3] Other investigators have reported a decrease in blood cholesterol during the administration of cortisone, but in most of these studies the number of cases and cholesterol determinations are so few as to prohibit definitive conclusions.[4-6] Adlersberg and his co-workers[2] administered ACTH and cortisone for longer periods of time than did Conn, and reported a marked increase in total serum cholesterol, especially of the ester fraction. Detailed data were given in only three cases all of which showed a decrease in cholesterol during the early phase of therapy followed by a steady rise. Wolfson and his associates[7] have also described a rise in cholesterol associated with continued use of cortisone which they attributed to depression of thyroid function. Because of the differing results offered in the literature, as well as the paucity of data upon which conclusions have been drawn, this study was conducted. We have followed the blood cholesterol levels in a series of patients during the administration of ACTH and cortisone and following the stress of surgery, and have compared the observed changes with variations seen in normal persons over similar periods of time.

METHODS AND RESULTS

Evaluation of the Cholesterol Method and Results Upon Normal Cases.—Total cholesterol and cholesterol esters were determined by the method of Hess[8] on the blood plasma of forty normal subjects and free cholesterol was 36.5 per cent of the total with a standard deviation of ± 4.8 per cent, a value practically the same as that previously obtained upon twenty-five normals (35.8 ± 4.7 per cent). While this ratio is somewhat higher than that reported by

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Schoenheimer and Sperry,[9] its constancy is in accord with their finding. It might be noted that the ratio found in the controls by Adlersberg and associates,[2] using the method of Schoenheimer and Sperry was 32.5 per cent, a value which is well within the range obtained by the procedure we have used. In order to determine the reproducibility of the results, it was shown that six separate determinations upon the same sample of blood gave an average ratio of 35.5 per cent with a maximum variation from the mean of −1.5 per cent while the total cholestrol averaged 154.6 mg. per 100 ml. of plasma with a maximum variation of −3.6 mg.

We have extended the study of the possible variations in this method of determining cholesterol by studying a series of normal cases over a period of hours and also over a period of days. The results of this study, for periods varying from two to eight hours, are given in Table I. The per cent change between the first and second determinations was calculated, and the average change in total cholesterol was +0.23 per cent, with a standard deviation of ±3.1. The average change in the free/total ratio was −0.06 per cent (S.D. ±2.6). Determinations were also made upon eleven subjects over periods from two to eight days, and the results are given in Table II. The per cent change between the two determinations was calculated for each individual, and the average change in total cholesterol was +2.6 per cent (S.D. ±7.8), while the average change in the F/T ratio was −6.2 per cent (S.D. ±3.6). One subject had six determinations over a period of eighty days, the average total cholesterol was 122 mg. per 100 ml. (S.D. ±5.4), the difference between the highest and lowest values being 13 per cent, the average of the F/T ratios was 35.9 per cent (S.D. ±1.2), and a maximum variation from the mean was +1.9 per cent.

It is apparent from these data that the total cholesterol values over a period of hours do not vary more than do replicate values upon the same sample, and that during periods of two to eight days there was not over 5 per cent variation either in total cholesterol or in the F/T ratio. These experiments serve to demonstrate what could be expected from the method, and also showed the degree of constancy of the values upon normal blood over a period of days. Turner and Steiner[10] reported similar experiments using the cholesterol method of Bloor, Pelkan, and Allen,[11] and they found a degree of constancy of values over twenty-four-hour periods and also over a period of one year quite similar to our results.

Administration of ACTH and Cortisone.—The control values for the concentration of total and free cholesterol in the plasma were determined for at least two days prior to treatment, and the values averaged. The lowest value obtained for the total cholesterol during treatment was taken for the comparison. The results following the administration

TABLE I. CHOLESTEROL DETERMINATIONS ON THE SAME INDIVIDUAL AFTER VARYING NUMBERS OF HOURS

NO.	HOUR	CHOLESTEROL (MG./100 ML.) TOTAL A	TOTAL B	CHANGE (PER CENT)	ESTER A	ESTER B	RATIO (F/T) A (PER CENT)	RATIO (F/T) B (PER CENT)	CHANGE (PER CENT)
1	2*	128	129	+0.8	79	76	38.3	41.0	+2.7
2	2	142	141	−0.7	92	89	35.2	36.9	+1.7
3	2	150	148	−1.3	95	95	36.6	35.8	−0.8
4	2	173	177	+2.3	123	120	28.9	32.2	+2.3
5	2	119	124	+4.1	67	68	43.7	45.1	+1.4
6	3	171	184	+7.6	100	119	41.5	36.0	−5.5
7	4	176	176	0.0	114	115	35.2	34.7	−0.5
8	4	163	166	+1.8	107	105	34.4	36.7	+2.3
9	5	226	224	−0.9	145	154	35.8	31.3	−4.5
10	6	158	156	−1.3	105	101	33.5	35.2	+1.7
11	6	134	131	−2.2	81	81	39.5	38.0	−0.5
12	8	138	132	−4.3	96	90	30.5	32.0	+1.5
13	8	136	132	−2.9	80	75	44.5	43.2	−1.3
Average				+0.23 ± 3.1†					−0.06 ± 2.6†

*Time interval between determinations A and B.
†Standard deviation.

of ACTH are given in Table III. In the nine cases the total cholesterol was reduced in each instance, and the range of reduction was from 9 to 33 per cent of the control values, with an average reduction of 24 per cent. The ester cholesterol was reduced to substantially the same extent, and there was no significant alteration in the ratio of free to total cholesterol. The least depression of serum cholesterol occurred in the two cases (No. 8 and No. 9) which showed the poorest physiological response to ACTH. Figs. 1 and 2 depict Cases 1 and 2 in Table III, and are given as examples of the changes produced. Eosinophil counts and 17-ketosteroid excretion, determined by the extraction method of Pincus[12] and the colorimetric procedure of Holtorff and Koch[13] are included to show the effectiveness of the doses of

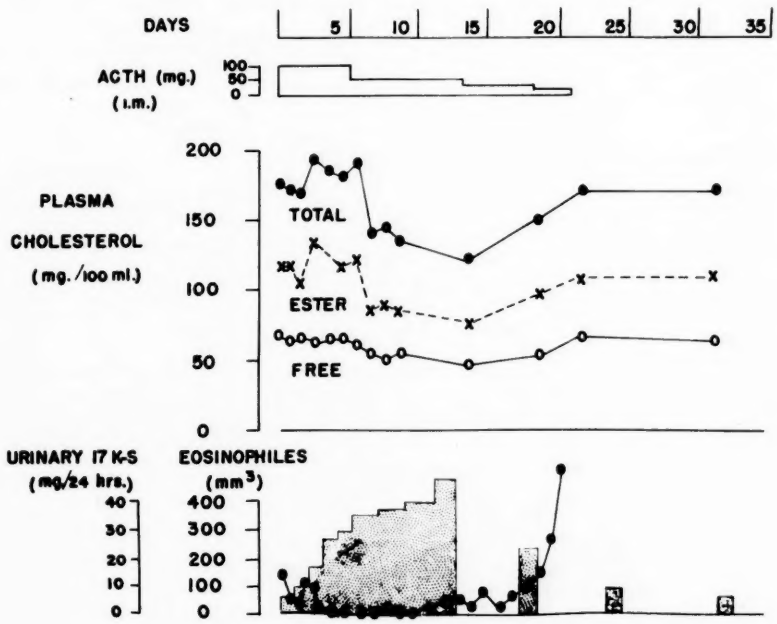

Fig. 1.—Effect of administration of ACTH. Case 3 of Table III.

TABLE II. CHOLESTEROL DETERMINATIONS UPON THE SAME INDIVIDUAL AFTER VARYING NUMBER OF DAYS

NO.	HOUR	CHOLESTEROL (MG./100 ML.) TOTAL A	TOTAL B	TOTAL CHANGE (PER CENT)	ESTER A	ESTER B	RATIO (F/T) A (PER CENT)	RATIO (F/T) B (PER CENT)	CHANGE (PER CENT)
1	2*	140	132	−5.7	91	82	35.0	38.0	+3.0
2	2	104	98	−5.7	62	55	40.3	43.8	+2.5
3	3	156	155	−0.6	95	92	39.1	40.6	+1.5
4	3	135	125	−3.7	88	82	34.8	34.4	−0.4
5	4	231	230	−0.4	145	160	36.8	30.4	−6.4
6	5	133	126	−5.2	87	81	34.6	35.7	+1.1
7	6	137	154	+12.4	106	121	22.6	21.4	−1.2
8	7	109	128	+17.4	69	77	36.7	39.9	+3.2
9	7	172	187	+8.0	118	125	31.4	33.1	+1.7
10	7	129	133	+3.1	77	79	40.2	40.6	+0.4
11	8	126	139	+9.3	75	89	43.8	36.0	−7.7
Average				+2.6 ± 7.8†					−0.2 ± 3.6†

*Time interval between determinations A and B.
†Standard deviation.

ACTH. The decrease in cholesterol usually started within three or four days after the administration of ACTH, and reached a maximum between the seventh and fourteenth days. The decrease was followed by a gradual return to approximately the control levels within ten to fourteen days. Blood cholesterol was determined five and six months after the ACTH was discontinued on Patient 3, and in both instances the values were the same as the control levels. One patient (Fig. 2) received ACTH intravenously for fourteen days after the cholesterol had begun to return to control levels, there having been refractoriness to the intramuscular treatment, and an immediate secondary decrease in cholesterol followed the

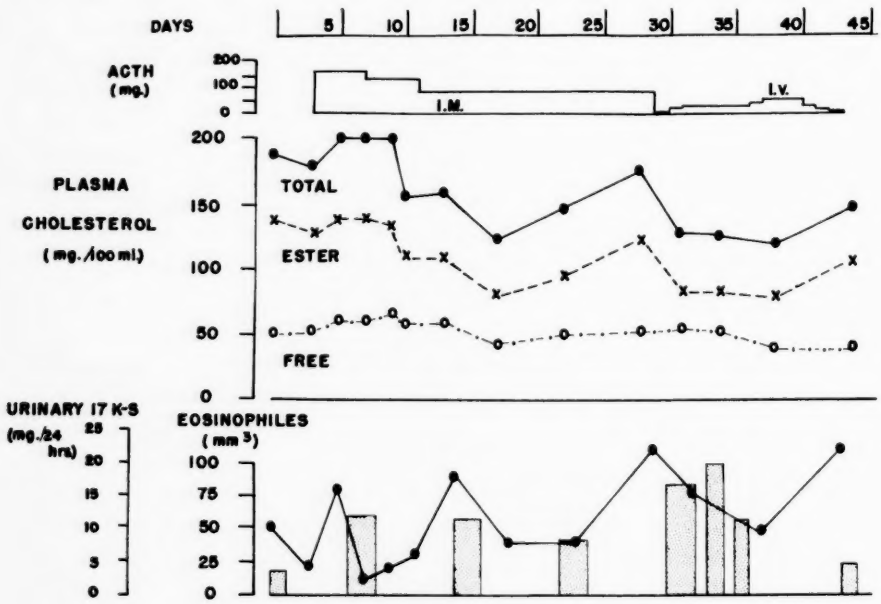

Fig. 2.—Effect of administration of ACTH. Case 2 of Table III.

changed route of administration of the drug. Of some interest in this patient, during intramuscular therapy, was the fact that the rise in cholesterol occurred concurrently with obvious clinical refractoriness to ACTH. Intravenous use of the drug resulted not only in a secondary drop in cholesterol, but also a very marked clinical response and increase in 17-ketosteroid excretion.

Two patients received ACTH intravenously for sixteen and twenty days, respectively. In both cases marked decreases in cholesterol occurred. One of these, a patient with scleroderma, shown in Fig. 3, had a sustained eosinopenia and also marked increases in the excretion of 17-ketosteroids and 11-oxysteroids. Prior to the administration of ACTH, the other patient had received 200 mg. of Compound F for twenty days, and during this time had shown a very slight decrease in cholesterol. In a short-term study of the comparative metabolic effects of Compound F and ACTH, Conn and his associates[14] noted that 17-hydroxycorticosterone failed to effect any significant depression of blood cholesterol.

The effect of cortisone on plasma cholesterol was less consistent than that of ACTH. Three cases responded with increases in total cholesterol and six cases showed a decrease, the algebraic average of the changes in the nine cases being a decrease of 7 per cent (Table IV). Fig. 4 illustrating Cases 7 and 8 in Table IV are typical examples of the less prominent changes produced by cortisone. It will be noted that the alterations in eosinophil count likewise are variable. It may well be that larger amounts of cortisone than those ordinarily

used are required to produce a result similar to that produced by ACTH. As an indication of this, it may be noted that Patient 1, shown in Fig. 5, showed a marked decrease in cholesterol following the administration of 200 to 300 mg. of cortisone—a dose larger than

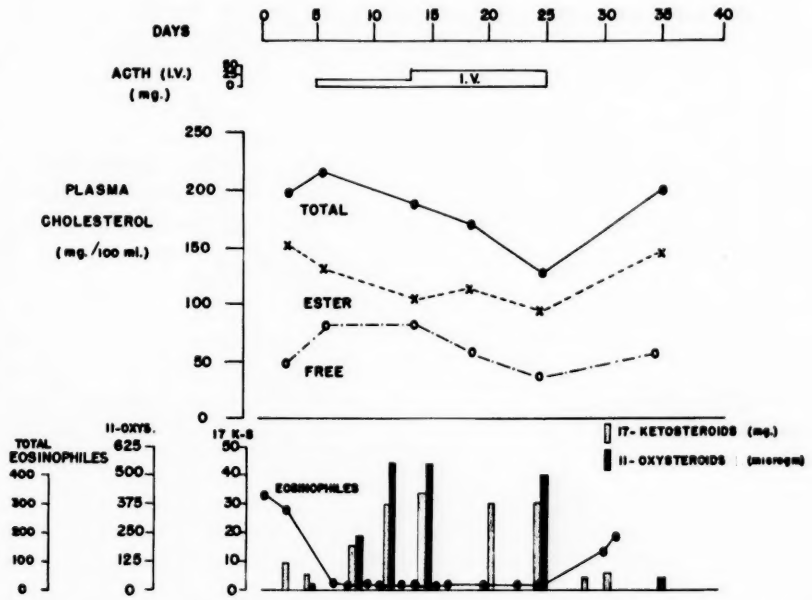

Fig. 3.—Effect of intravenous administration of ACTH. 11-oxysteroids are expressed in terms of formaldehyde.

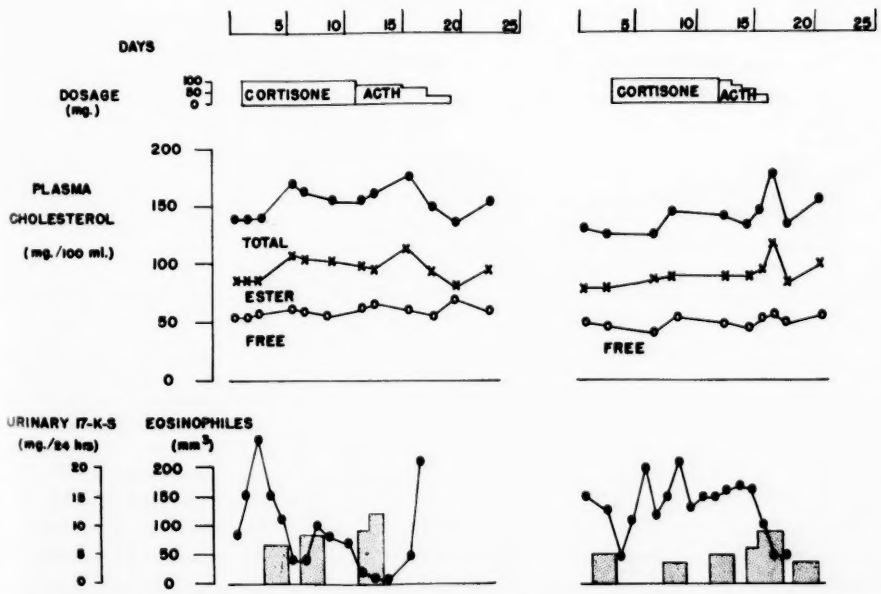

Fig. 4.—Effect of administration of cortisone and ACTH. Cases 7 and 8 of Table IV, which are also Cases 1 and 2 of Table V.

TABLE III. EFFECT OF ACTH ON PLASMA CHOLESTEROL
CHOLESTEROL (MG. PER 100 ML.)

PATIENT	TOTAL			ESTER			F/T RATIO		
	CONTROL	RX	PER CENT CHANGE	CONTROL	RX	PER CENT CHANGE	CONTROL	RX	CHANGE
1. Chronic gout	176	120 (13*)	−32	111	72	−35	36.3	40.0	+3.7
2. Psoriasis	184	124 (14)	−33	133	82	−39	27.7	34.7	+7.0
3. Arthritis	197	148 (7)	−25	139	104	−25	29.5	29.7	−0.2
4. Leukemia	172	137 (7)	−20	115	90	−22	33.1	34.3	+1.2
5. Arthritis	174	120 (8)	−31	116	80	−31	33.3	33.3	0.0
6. Periarteritis nodosa	157	108 (8)	−31	100	65	−35	36.3	39.8	+3.5
7. Scleroderma	117	92 (6)	−21	82	58	−21	35.0	36.9	+1.9
8. Arthritis	156	138 (7)	−12	94	88	− 6	39.7	36.2	−3.5
9. Leukemia	195	178 (8)	− 9	131	120	− 8	32.8	32.6	−0.2
Average			−24 ± 8.7†			−25			+1.5 ± 2.6†

Cases are arranged in order of degree of adrenal stimulation as measured by 17-ketosteroid excretion.

*Day of maximal depression after institution of therapy.

†Standard deviation.

TABLE IV. EFFECT OF CORTISONE ON PLASMA CHOLESTEROL
CHOLESTEROL (MG. PER 100 ML.)

PATIENT	TOTAL			ESTER			F/T RATIO		
	CONTROL	RX	PER CENT CHANGE	CONTROL	RX	PER CENT CHANGE	CONTROL	RX	CHANGE
1. Psoriasis	195	146	−25	127	85	−33	34.9	41.7	+6.8
2. Leukemia	173	136	−21	122	94	−23	29.5	30.8	+1.3
3. Arthritis	156	138	−12	106	90	−15	32.7	34.8	+2.1
4. Scleroderma	126	115	− 8	84	82	− 1	33.3	28.8	−4.5
5. Arthritis	158	161	+ 2	104	105	0	32.9	34.7	+1.8
6. Arthritis	155	150	− 3	99	100	+ 1	36.1	33.3	−2.8
7. Arthritis	125	130	+ 4	75	85	+13	40.0	34.6	−5.4
8. Arthritis	140	155	+10	85	100	+18	39.3	35.5	−3.8
9. Leukemia	231	210	− 9	153	137	−10	33.8	34.8	+1.0
Average			−7± 13.4*			−6			−0.4 ± 4.0*

Patients are listed in order of dose and degree of physiological stimulation.

*Standard deviation.

TABLE V. EFFECT OF ACTH ON PLASMA CHOLESTEROL FOLLOWING A COURSE OF CORTISONE THERAPY
CHOLESTEROL (MG. PER 100 ML.)

	TOTAL			ESTER			F/T RATIO		
	CONTROL	RX	PER CENT CHANGE	CONTROL	RX	PER CENT CHANGE	CONTROL	RX	CHANGE
1. Arthritis	161	170 (4*)	+ 6	105	115	+10	34.8	32.4	−2.4
2. Arthritis	140	175 (4)	+25	90	120	+33	37.8	31.4	−6.4
3. Arthritis	157	175 (6)	+12	102	115	+13	35.0	34.3	−0.7
4. Leukemia	191	210 (7)	+ 9	125	137	+ 8	34.5	34.9	+0.5
5. Leukemia	136	178 (4)	+31	94	120	+28	30.9	32.5	+1.6
Average			+17 ± 10.9†			+17			−1.5 ± 2.1†

*Number of days following start of ACTH.

†Standard deviation.

the 100 mg. daily administered to the other patients. One other patient who showed a significant drop in serum cholesterol, Case 2, in Table IV, obtained more evidence of physiologic stimulation than the other patients treated.

Five patients were given ACTH immediately following the administration of cortisone; the initial response in each instance was a rise in total cholesterol. These results are shown in Table V, and Fig. 4 indicates a typical response. Conn and associates[1] noted that a rise in cholesterol sometimes occurred after cortisone treatment was stopped, and the increase we observed when ACTH was given after a course of cortisone may have been the continuation of the cortisone effect. However, since adequate eosinopenia and a substantial increase in 17-ketosteroid excretion occurred soon after the ACTH was started, it is more likely that the increase noted was related to an ACTH effect. This initial rise in cholesterol was usually followed by a decline.

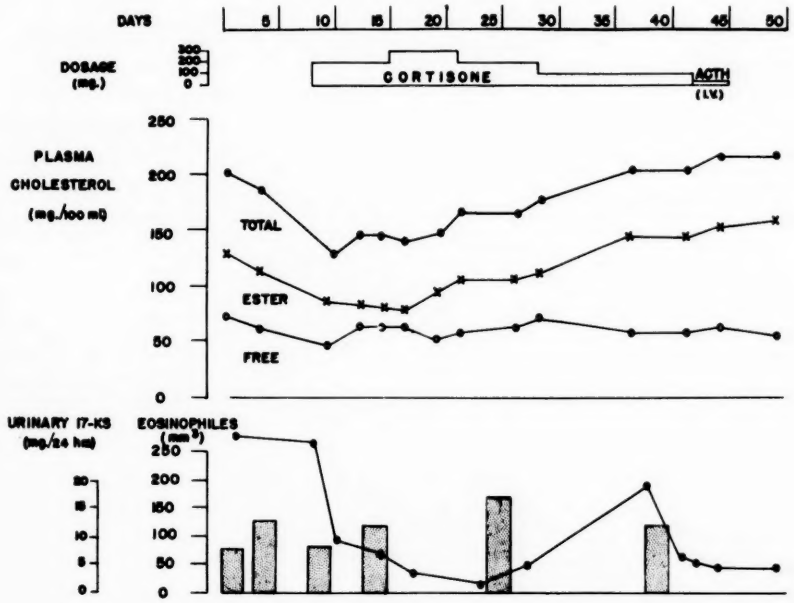

Fig. 5.—Effect of the administration of cortisone. Case 1 of Table IV.

In Table VI are given the data on the changes in plasma cholesterol and eosinophil count following surgery. Typical examples of the effect are presented in Fig. 6 of Cases 3 and 7 in Table VI. In nine of the ten cases there was significant reduction of the total cholesterol, and in the cases which showed the most marked drop there was a substantial rise in the F/T ratio. The other patient was studied only during the first postoperative day. The decrease occurred in six of the nine cases between twenty-four and forty-eight hours following surgery, whereas, the eosinopenia reached its peak within twelve hours. The decrease in blood lipids, including cholesterol, twenty-four hours following surgery, have been reported by Mann, Bettcher, Cameron, and Peters.[15] They were of the opinion that since all the lipid components were proportionately affected without distortion, the decreases were not due to a liver disturbance. It might be noted, however, that Tagnon, Robbins, and Nichols[16] found a significant increase in the retention of bromsulfalein immediately after extra-abdominal surgery, indicating, in their opinion, definite impairment of liver function. Keeton and his co-workers,[17] in a more complete study of the effects of elective surgery on nitrogen balance and hepatic function, noted an immediate impairment of bromsulfalein removal with return to normal by the fifth or later postoperative day. They also demonstrated an increase in urinary urobilinogen excretion, maximal on the third postoperative day, with a return to

normal only after the sixth day. Similar to the observations reported here these workers found a significant reduction in total cholesterol and cholesterol ester. This occurred only on the third postoperative day, but from the tabulated data presented it appears as though no cholesterol determinations were made prior to that day. The postulated causes of hepatic insufficiency during the early postoperative period, offered by these authors, do not include the possible effect of increased adrenal cortical secretion.

Four cases of bronchial asthma were given a single dose of ACTH and their cholesterol levels and eosinophil counts were followed over a period of several days. The cholesterol values, as shown in Table VII, decreased markedly in three of the four cases, the decrease being mainly in the ester fraction. In the patient who showed no drop in cholesterol, Case 4, no determinations were made after the first twenty-four hours following injection. The results on Cases 2 and 3 are shown in Fig. 7, and it may be noted that the eosinopenia occurred within several hours after the ACTH was given, whereas the decrease in cholesterol did not occur until at least forty-eight hours later. The time relationships are therefore very similar to those occurring after surgical stress, and the change in F/T ratio was also more comparable with the effects of surgery.

Table VIII summarizes the average alterations in total cholesterol and in the F/T ratio in the various groups of patients which were studied.

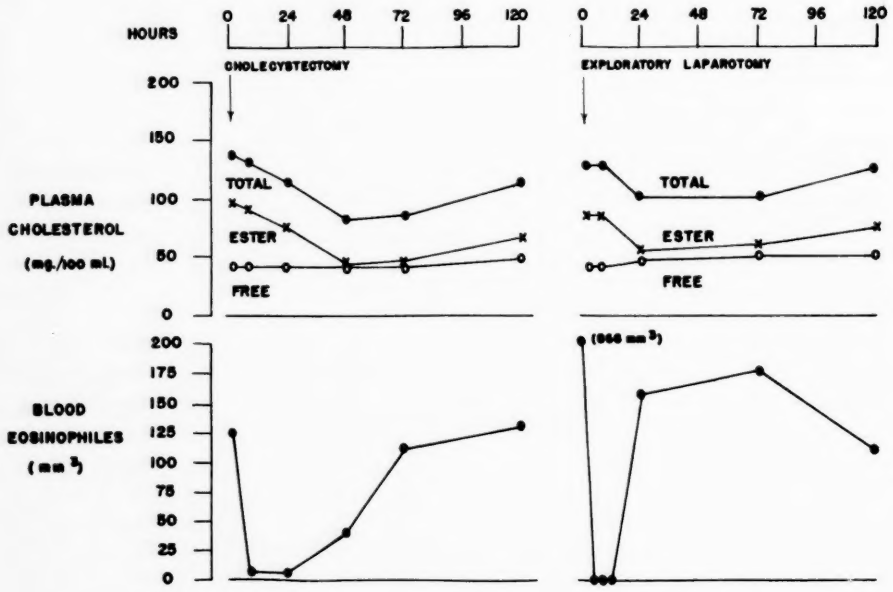

Fig. 6.—Effect of surgical stress. Cases 3 and 7 of Table VI.

DISCUSSION

Because of the fact that a diminution of adrenal cholesterol has been shown to occur following adrenal stimulation and for the reason that there exists considerable evidence that some steroid hormones are formed from cholesterol ester in the body,[18] it appeared logical to Conn that the drop in serum cholesterol, especially of the ester fraction, which followed ACTH resulted from increased utilization of cholesterol to form adrenal hormones. The alternative possibility, namely, that the reduction in cholesterol was secondary to hepatic dysfunction, was considered to be extremely unlikely, in view of the fact that

TABLE VI. EFFECT OF OPERATIVE STRESS ON PLASMA CHOLESTEROL
CHOLESTEROL (MG. PER 100 ML.)

OPERATION	TOTAL			ESTER			F/T RATIO		
	CONTROL	POSTOP.	PER CENT CHANGE	CONTROL	POST-OP.	PER CENT CHANGE	CONTROL	POST-OP.	CHANGE
1. Thyroidectomy	119	134 (24*)	+13	67	79	+18	43.7	41.0	− 2.7
2. Cholecystectomy	171	142 (72)	−17	100	90	−10	41.5	36.6	− 4.9
3. Cholecystectomy	138	82 (48)	−40	96	42	−56	40.6	48.8	+ 8.2
4. Gastric resection	126	112 (48)	−11	70	–	–			
5. Supracondylar amputation	150	110 (12)	−26	95	65	−32	36.6	41.0	+ 4.4
tumor	165	142 (24)	−14	95	80	−16	42.5	43.7	+ 1.2
6. Removal of thymic		101 (24)	−21	85	55	−35	33.6	45.5	+11.9
7. Adrenal biopsy	128								
8. Exploratory lap.	128	117 (24)	− 9	86	–	–			
9. Resection of colon	121	93 (8)	−23	73	51	−30	39.8	45.2	+ 5.4
10. Thyroidectomy	132	113 (24)	−14	79	63	−20	40.1	44.2	+ 4.1
Average			−16 ± 11.0†			−23			+2.8 ± 4.9†

*Figure in parentheses is hours following operation.
†Standard deviation.

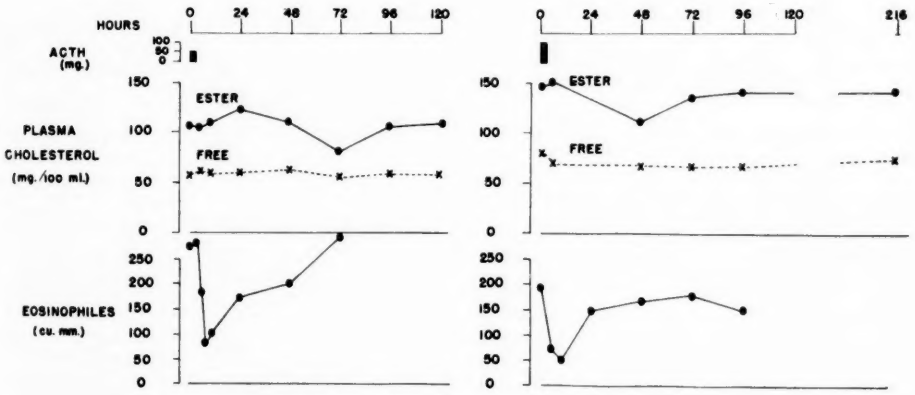

Fig. 7.—Effect of single doses of ACTH. Cases 2 and 3 of Table VII.

TABLE VII. EFFECT OF A SINGLE INJECTION OF ACTH IN PATIENTS WITH BRONCHIAL ASTHMA
CHOLESTEROL (MG. PER 100 ML.)

NO.	ACTH (MG.)	TOTAL			ESTER			F/T RATIO		
		CONTROL	RX	PER CENT CHANGE	CONTROL	RX	PER CENT CHANGE	CONTROL	RX	CHANGE
1	100	134	115 (72*)	−14	81	62	−23	39.6	46.1	+6.5
2	100	226	180 (48)	−20	145	112	−23	35.8	37.7	+1.9
3	50	163	135 (72)	−17	106	80	−24	35.0	41.5	+6.5
4†	50	157	153 (24)	− 2	105	100	− 5	33.0	34.6	+1.6
Average				−16 ± 8.5‡			−19			+4.1 ± 2.7‡

*Number of hours after ACTH was given at time of maximum cholesterol decrease.
†Had no bloods studied after twenty-four hours.
‡Standard deviation.

there was little decrease in blood cholesterol following the administration of cortisone. However, the factor of dose response could readily explain this discrepancy, for it has become apparent that the amounts of cortisone ordinarily used to obtain therapeutic effect lacked the physiologic potency of the dose of

ACTH considered comparable on a clinical basis. Our data suggest that the more marked physiologic stimulation resultant from cortisone was associated with significant depression of the serum cholesterol, and in the one patient who was given large amounts of cortisone, the depression of blood cholesterol was equal to the average decrease which accompanied ACTH therapy.

In one patient suffering from the intrahepatic type of obstructive jaundice, not included in the tabulated data, there was, prior to the administration of ACTH, a marked depression of cholesterol ester with a F/T ratio of 54.8 per cent. The use of ACTH, although associated with definite evidence of adrenal stimulation, was not accompanied by any further depression of cholesterol ester. Presuming that the hepatic insufficiency in this patient would not be worsened by ACTH, one would not expect ACTH induction of further change in the cholesterol level if such were due to hepatic insufficiency, while if the cholesterol ester were used to form adrenal steroid hormone one would have expected the usual depression to occur. Similarly, the drop in cholesterol

TABLE VIII. COMPARISON OF VARIATION IN BLOOD CHOLESTEROL IN DIFFERENT GROUPS

TYPE OF PATIENT	NO. OF CASES	AVERAGE CHANGE IN TOTAL CHOLESTEROL	AVERAGE CHANGE F/T RATIO
		%	%
I. Normal (after a period of hours)	13	+ 0.23 ± 3.1*	−0.1 ± 2.6*
II. Normal (after a period of days)	11	+ 2.6 ± 7.8	−0.2 ± 3.6
III. Continuous ACTH administration	9	−24.0 ± 8.7	+1.5 ± 2.6
IV. Continuous cortisone administration	9	− 7.0 ± 13.4	−0.4 ± 4.0
V. ACTH following cortisone	5	+17.0 ± 10.9	−1.5 ± 2.1
VI. Following surgical stress	10	−16.0 ± 11.0	+2.8 ± 4.9
VII. Single injection of ACTH	4	−16.0 ± 8.5	+4.1 ± 2.7

*Standard deviation.

after the administration of a single dose of ACTH followed by a significant length of time, the period of maximal adrenal stimulation, and occurred even later than the period during which the adrenal might be expected to replenish its depleted cholesterol stores. The time of cholesterol decrease in these patients did, however, correspond well with the time of decrease which occurred after the stress of surgery, and equally well with the period of maximal hepatic insufficiency which occurs after operative trauma.

The rise in serum cholesterol which occurred in patients given ACTH after a course of cortisone is difficult to explain on the basis of either hepatic insufficiency or increased adrenal activity. However, in view of the obvious evidence of increased adrenal activity in these cases, this observation offers some evidence against the concept that a drop in blood cholesterol is secondary to increased cholesterol utilization by the adrenal gland.

In view of these findings it is difficult to be entirely satisfied with the explanation that the decrease in serum cholesterol which follows ACTH is the result of the utilization of blood cholesterol for the formation of steroid hormones. On the other hand, it is difficult to reconcile a possibility of a decrease in cholesterol on the basis of hepatic insufficiency with the evidence of improvement in liver function seen in patients with hepatitis treated with ACTH.[19] In the

patients treated with ACTH the change in cholesterol ester was more striking but there was in most instances a decrease in free cholesterol sufficient in amount to preserve the usual F/T ratio at the control level. This fact speaks against hepatic change as a causative factor in the cholesterol change following ACTH. In many of the surgically traumatized individuals there was a marked decrease in the ester fraction, with a rise in the F/T ratio, and in these cases it is not unlikely that the changes in cholesterol were the result of temporary impairment of hepatic function.

Thus there appear to be basic weaknesses in attempts to explain the decrease in serum cholesterol following ACTH on the basis of either conversion of blood cholesterol to cortical hormones or interference with cholesterol metabolism in the liver. There remain two major possibilities that might explain this serum cholesterol change, namely, increased loss or decreased formation. There exists no evidence that the excretion of cholesterol is accentuated by ACTH but the possibility of impairment of synthesis deserves consideration.

The demonstration by Srere and his co-workers[20] that surviving adrenal cortical tissue can synthesize cholesterol from acetate, suggests that while adequate supplies of cholesterol are essential for the formation of cortical hormone, such may be synthesized locally and not necessarily be obtained from the blood in the form of cholesterol ester. The glyconeogenetic properties of certain of the adrenal hormones, namely, those oxygenated at the 11th carbon atom, have been well established by several investigators. Sayers and his group[21] found that ACTH produced an increase in liver glycogen and a decrease in adrenal cholesterol and ascorbic acid. Although the fall in ascorbic acid was immediate, the decrease in cholesterol reached its maximum in about twelve hours after the injection of ACTH, approximately at the time of maximum increase in liver glycogen. It is the opinion of Long,[22] among many other investigators, that the glyconeogenesis results from protein breakdown. As a consequence of the diversion of protein into the channels of carbohydrate metabolism, there will be a diminution of the amount of amino acids available for cholesterol synthesis, which Bloch and Rittenberg[23] and Zabin and Bloch[24] have shown can be formed from acetate and even to a greater extent from isovalerate, both of which are amino acid metabolites. Consequently there exists the possibility that the reduction in blood cholesterol is the result of lack of amino acids necessary for its synthesis because of increased protein metabolism induced by adrenal hormone activity. It has been suggested by Ingle[25] and also by Segaloff and Many[26] that fat as well as protein may be the source of the glucose made by the body under the influence of the adrenal hormones. Since acetic acid is likewise a metabolite of fat, the diversion of fat to carbohydrate would also tend to reduce the amount of the precursors of cholesterol.

SUMMARY AND CONCLUSIONS

1. The determination of plasma cholesterol and cholesterol esters by chromatographic separation is reproducible within 2 per cent on replicate determinations on the same sample. The variation in plasma cholesterol content on

the same patient in periods of time varying from two to eight hours is likewise within 2 per cent, and for periods of time from two to eight days the variation is approximately 5 per cent.

2. The administration of ACTH in nine patients caused an average reduction of total cholesterol of 24 per cent, and in general the greater decrease in cholesterol was well correlated with greater physiologic stimulation by ACTH. In two patients who obtained little stimulation from the ACTH there was no significant depression of cholesterol. Treatment with ACTH caused no significant change in the ratio of free to total cholesterol. Intravenous administration of ACTH to one patient for 20 days, and to another for fourteen days produced a rapid and marked decrease in plasma cholesterol.

3. The effect of the administration of cortisone to nine patients was less consistent than that of ACTH. The average change for all nine cases was a decrease of 7 per cent, but a more marked decrease was observed in two patients who were given larger doses of cortisone.

4. ACTH was administered to five patients immediately after the treatment of cortisone ceased and in each instance there was an initial rise in total plasma cholesterol.

5. Compound F was given to one patient for twenty days, producing only a slight decrease in plasma cholesterol, although a marked decrease in the eosinophil count occurred.

6. In nine of ten cases there was a decrease in plasma cholesterol following surgery, the decrease in all instances coming on quite later than the time of reduction in eosinophil count, and associated, in the cases showing the more marked decrease, with a change in the F/T ratio indicative of inproportionate lowering of the cholesterol ester fraction. A single injection of 50 or 100 mg. of ACTH produced a reduction of cholesterol maximal forty-eight to seventy-two hours after the ACTH was administered.

7. It is suggested that the decrease in blood cholesterol which accompanies the administration of ACTH is caused by the direct action of adrenal hormone either by inducing temporary impairment of hepatic function, or, more probably, by supressing cholesterol synthesis.

A portion of the ACTH used in this study was furnished by Dr. John R. Mote and by Dr. Harley E. Cluxton, Jr., formerly of the Armour Laboratories.

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STUDIES IN SERUM PROTEINS

VI. The Extension of the Standard Biuret Method to the Estimation of Total Protein in Urine

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AN INCREASED urinary excretion of protein commonly accompanies the abnormal serum protein fractionation patterns found in a number of disorders, such as disseminated lupus erythematosus, multiple myeloma, nephrotic syndrome, and the nephritides. In following such patients, laboratories more usually engaged in studies of serum protein structure often find it desirable to estimate proteinuria by a technique more accurate than the rough procedures commonly used for clinical urinalysis. In earlier reports, the use of a single standardized stable biuret reagent and a single calibration curve in a rapid micromethod for the estimation of the five major serum protein component has been described,[1, 2] and a subsequent report will describe its application to gamma-globulin subfractionation.[3] The ability of these simple methods to give serum protein fractionation patterns comparable to those obtained by electrophoretic analysis has been confirmed by a number of investigators[4-7] and these techniques have been applied to a number of problems.[8-11]

The present report describes a simple method for the quantitative estimation of urine protein with the same stable biuret reagent and standard calibration curve (or extinction coefficient) employed in the rapid chemical micromethod for serum protein fractionation.†

METHOD

Reagents.—Biuret reagent, Weichselbaum, modified: Prepare an accurately titrated 0.2N NaOH solution. In a two-liter volumetric flask, dissolve 90 Gm. of Rochelle salt (potassium sodium tartrate) in about 400 ml. of the 0.2N NaOH solution. When the Rochelle salt has dissolved add 10 Gm. of $CuSO_4 \cdot 5H_2O$. After dissolving the copper sulfate, add 10 Gm. of KI and dilute to two liters with 0.2N NaOH. Filter and store in a rubber-stoppered Pyrex bottle. This reagent is stable for many months at room temperature.

10 per cent trichloracetic acid.

Preparation of the Standard Curve.—

1. The standard curve used is that which is prepared for the estimation of total protein in serum, as described below. This curve is also used for all other determinations in

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†Only that portion of the fractionation procedure essential for urine protein determinations is given below. Copies of the complete method are available upon request to the authors.

this series.[1, 2] As a standard protein solution, any of the following are convenient:

(a) A sample of pooled normal human serum, the protein content of which has accurately been determined by Kjeldahl analysis.

(b) Commercial human albumin solution (Cutter Laboratories). This material has the advantage of permitting the preparation of standards with protein concentrations well above those ordinarily present in normal sera. While expensive, it is supplied in an accurately standardized concentration of 25 Gm. per 100 milliliters.

(c) A study by Levin and Brauer[12] suggests bovine serum albumin, Armour, to be an acceptable substitute for human serum albumin.

2. Prepare standard protein solutions with concentrations of 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 Gm. per 100 ml. by diluting the standard protein with appropriate amounts of normal saline. Determine the colorimeter readings obtained when each of these standard solutions is carried through the method for serum total protein, as described below. From the data so obtained, construct the standard colorimeter curve.

3. The Evelyn photoelectric colorimeter has been used in these studies. For this instrument, using the 10 ml. aperture and the 540 millimicra filter, with the reagent blank adjusted to 100 per cent transmission, the following equation relates the per cent transmission (G) of an unknown sample to the amount of protein contained in an aliquot of the sample:

Protein concentration, mg. per aliquot = (2—log G) /.0440.

Estimation of Total Protein in Serum or Standard Solutions.—

1. Pipette 0.4 ml. of serum or standard protein solution into a 10 ml. volumetric flask or graduated mixing cylinder, and dilute to 10 ml. with distilled water. Mix well by inversion, and pipette 5.0 ml. of this diluted material into an Evelyn cuvette.*
2. Pipette 5.0 ml. of biuret reagent into the cuvette and mix by swirling.
3. Prepare a blank with 5.0 ml. of distilled water and 5.0 ml. of biuret reagent.
4. Let the solutions stand at least thirty minutes to permit full color development. Once color has fully developed, it is exceptionally stable. Readings may be deferred for as long as twenty-four hours.
4. With the 10 ml. aperture and 540 millimicra filter in place, read on the photoelectric colorimeter with the blank set at 100 per cent transmission.

Estimation of Total Protein in Urine.—

1. To a 15 ml. graduated Pyrex centrifuge tube, add 5 ml. of clear, filtered urine, 5 ml. of 10 per cent trichloracetic acid, and 5 ml. of distilled water. Mix by inversion.† Centrifuge for ten to fifteen minutes at 1,500 to 2,000 r.p.m. or longer if the supernatant is not clear at the end of this period. When urines contain more than 0.3 per cent protein (6 Gm. per 2,000 ml.), they will require preliminary dilution or reduction in the size of the aliquot used.
2. Decant off and discard the supernatant. Permit the tube to drain by inversion for a minute or two, then carefully wipe off any liquid remaining on the lip of the tube.‡
3. Add 5 ml. of biuret reagent to the centrifuge tube. With a pointed stirring rod, stir the mixture until the precipitate dissolves. Rinse the stirring rod with distilled water and dilute the mixture to 10 ml. with distilled water. When the solution is completely clear, decant into an Evelyn cuvette.
4. Prepare a blank with 5 ml. of distilled water and 5 ml. of biuret reagent.

*If 0.4 ml. pipettes are unavailable, pipette 0.2 ml. of serum into an Evelyn cuvette and add 4.8 ml. of distilled water.

†When handling twenty-four hour urines, calculations are facilitated if those urines with a volume less than two liters are routinely adjusted to 2,000 ml. with distilled water before determinations are undertaken. When the twenty-four hour urine has a greater volume, it is convenient to adjust the volume to the nearest even 100 milliliter.

‡Washing the precipitate at this point has been found to provide no significant alteration either in apparent urine protein concentration or in the recovery of added protein, and is therefore omitted.

TABLE I. RECOVERY OF ADDED SERUM ALBUMIN, WHOLE SERUM, AND SERUM GAMMA GLOBULIN FROM URINES OF VARYING PROTEIN CONTENT

PATIENT, DATE, AND DIAGNOSIS	TYPE OF PROTEIN ADDED TO URINE	URINE PROTEIN (MG./5 ML.)	ADDED PROTEIN (MG./5 ML.)	EXPECTED VALUE (MG./10 ML.)	VALUE FOUND (MG./10 ML.)	PROTEIN RECOVERED (PER CENT)
Mr. G.: 6/28/51 Laurence-Moon-Biedl syndrome	Human serum albumin	0.23	10.14	10.37	10.95	105.7
Mr. J.: 6/8/51 Rheumatoid arthritis	Human serum albumin	0.15	10.14	10.29	11.19	108.9
Mr. W.: 7/10/51 Rheumatoid arthritis	Human serum albumin	0.15	10.14	10.29	11.09	107.9
Miss J.: 4/26/51 Diss. lupus erythematosus	Human serum albumin	3.78	9.98	13.76	14.44	106.9
Miss J.: 5/1/51 Diss. lupus erythematosus	Human serum albumin	3.17	9.98	13.15	13.86	106.9
Miss J.: 5/7/51 Diss. lupus erythematosus	Human serum albumin	6.62	9.98	16.60	17.00	104.1
Miss J.: 5/14/51 Diss. lupus erythematosus	Human serum albumin	8.66	9.98	18.64	18.74	101.0
Miss J.: 5/20/51 Diss. lupus erythematosus	Human serum albumin	8.40	9.98	18.38	18.74	103.5
Miss J.: 5/27/51 Diss. lupus erythematosus	Human serum albumin	10.65	9.98	20.63	20.99	103.5
Miss J.: 6/3/51 Diss. lupus erythematosus	Human serum albumin	5.88	10.34	16.22	16.89	106.5
Miss J.: 6/10/51 Diss. lupus erythematosus	Human serum albumin	6.97	10.34	17.31	17.37	100.6
Miss J.: 6/19/51 Diss. lupus erythematosus	Human serum albumin	13.86	10.34	24.20	23.48	93.0
Miss J.: 6/24/51 Diss. lupus erythematosus	Human serum albumin	8.14	10.34	18.48	18.60	101.2
Miss J.: 7/1/51 Diss. lupus erythematosus	Human serum albumin	10.65	10.34	20.99	20.85	98.6
Miss J.: 7/29/51 Diss. lupus erythematosus	Human serum*	9.14	14.24	23.38	22.80	95.9
Miss J.: 8/5/51 Diss. lupus erythematosus	Human gamma globulin	5.99	10.41	16.40	16.31	99.1
Mr. S.: 12/10/51 Chronic glomerulonephritis	Human serum albumin	5.00†	9.16	14.16	13.98	98.0
Mr. S.: 12/11/51 Chronic glomerulonephritis	Human serum albumin	4.33†	9.16	13.49	13.39	98.9
AVERAGE RECOVERY OF ADDED PROTEIN = 102.2%, STANDARD DEVIATION = ±4.4%						

*The serum used was from a young man apparently healthy except for retrobulbar neuritis. It was diluted 1:20 before being mixed with the urine.

†A preliminary 1:5 dilution of urine was required to bring the final color developed into the optimal colorimeter range. The actual concentrations of protein in the undiluted urines were: 0.50 per cent on 12/10/51 and 0.43 per cent on 12/11/51.

5. Let color develop for at least thirty minutes. Then read at 540 millimicra on the photoelectric colorimeter, with the 10 ml. aperture in place and the blank set at 100 per cent transmission.
6. *Calculations:*
   (a) If the equation relating protein concentration and galvanometer readings given above is used, the amount of protein in a 5 ml. aliquot of urine is obtained directly.
   (b) If the standard serum protein curve is used, obtain the apparent protein concentration from the colorimeter readings. Then, since a 5 ml. urine aliquot has been used rather than the 0.2 ml. serum aliquot for which the curve was prepared, divide the protein concentration obtained from the standard curve by 25 to obtain the concentration of protein per 100 ml. of urine.

EXPERIMENTAL

Inadequacy of Biuret Method for Estimation of Urine Protein Without Prior Precipitation.—In preliminary studies, the possibility of estimating urine protein by the biuret method without preliminary protein precipitation was examined. This method has an attractive simplicity, although, in practice, its apparent advantages are largely offset by the necessity of introducing a correction for the light absorption of urine itself at 540 millimicra. However, even with such a correction, it was found that this type of procedure regularly yielded recoveries of added albumin which were grossly excessive and averaged 120 to 130 per cent of the added protein. Such high recoveries obviously rendered the direct biuret method inapplicable; a possible cause of these errors is discussed below.

Recovery of Added Protein after Trichloracetic Acid Precipitation.—Table I summarizes the recovery of added normal human serum albumin, normal human serum gamma-globulin, and normal human whole serum from urines containing little protein, and from those containing significant amounts of Bence-Jones protein or other serum proteins. In the experiments summarized, the recovery of added protein averaged 102.2 per cent (S.D. ± 4.4 per cent). The recoveries of whole serum or gamma-globulin are similar to those obtained with serum albumin. There is some slight tendency to high recovery of added serum albumin. The magnitude of this deviation is so small as to be of little practical importance, but its cause is of considerable theoretical interest.

It seems reasonable to assume that the factor responsible for the slightly high recoveries of added protein after trichloracetic acid precipitation is also the source of the greatly excessive recoveries obtained in attempts to estimate urine total protein by the biuret reagent without prior protein precipitation. If this is granted, one may exclude the possibility that the slightly high recoveries after precipitation are due to co-precipitation of a polypeptide component which gives the biuret reaction but is not precipitated by trichloracetic acid unless a large amount of protein is present. This possibility is also rendered improbable by a failure to observe supernormal recoveries of albumin which was added to urine, the polypeptide content of which had been increased by prior addition of ACTH or peptone.

A more attractive possibility is that urine contains some constituent which does not itself give color in the biuret reaction but which combines with added

centrations of protein in the undiluted urines were: 0.50 per cent on 12/10/51 and 0.43 per cent on 12/11/51.

proteins to give a complex which produces an unusually large amount of color per milligram of protein. As an incidental observation, it has been noted that the addition of gentisic acid to urine in concentrations of 20 mg. per 100 ml. or more causes an increase in the apparent protein concentrations obtained with the biuret reagent. Perhaps normal urinary phenols which behave in a manner similar to gentisic acid are responsible for the high recoveries noted when urine protein is estimated by the direct biuret method, and to a lesser extent after trichloracetic acid precipitation.

Effect of Other Variables on Recoveries.—The amount of protein precipitated by 5 or 10 per cent sulfosalicylic acid averaged 91 per cent of that precipitated by trichloracetic acid in three experiments.* In addition, the precipitate obtained with sulfosalicylic acid dissolved only with great difficulty. The desirability of diluting the urine prior to precipitation was suggested by slight decreases in recoveries obtained when 10 per cent trichloracetic acid was added to undiluted high-protein content urine. However, a 1:1 dilution, as used in the present method, appeared to be an adequate safeguard. Higher dilutions did not alter apparent protein concentration significantly. The effects of added glucose in various concentrations and that of carrying out the procedures within a fairly wide range of room temperatures were studied and found insignificant.

SUMMARY

Urine total protein may be estimated accurately by the biuret method following a preliminary precipitation with trichloracetic acid. The biuret reagent recommended and standard curve employed are identical with those previously utilized in a rapid micromethod for serum protein fractionation. Recoveries of protein added to urine averaged 102.2 per cent (S.D. ± 4.4 per cent).

Attempts to estimate urine protein by the biuret reaction without preliminary protein precipitation were unsatisfactory because of grossly excessive recoveries of added protein. The preliminary protein precipitation reduces this error to a value which is of little practical consequence, but it does not completely abolish the error. One possible source of supernormal recoveries of added protein is indicated, but the actual cause has not been definitely established.

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*Older reports that a portion of the urinary protein fails to be precipitated by trichloracetic acid were confirmed by Beckman, Hiller, Shedlovsky, and Archibald.[13] The acid-soluble protein was found to comprise 4 per cent to 20 per cent of the urine protein and to have the electrophoretic mobility of an alpha-globulin when studied in pH 8.6 veronal buffer, and was precipitable by acetic acid and heat or by half-saturation with ammonium sulfate. In these characteristics, it resembles the perchloric acid-soluble alpha-mucoproteins of serum recently studied by Winzler and others.[14] The original communication of Beckman and his associates noted the occurrence of similar material in serum.

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THE DRAINAGE OF RADIOACTIVE SILVER COLLOIDS BY THE LYMPHATICS FOLLOWING INTRAPULMONARY ADMINISTRATION IN DOGS

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IT HAS been shown elsewhere[3] that when radioactive gold colloids are instilled in the lung by tracheal tube or bronchoscope the fluid vehicle is rapidly absorbed, leaving the radioactive inert material uniformly distributed in the lung alveoli. The gold colloid remains for a considerable number of days and may thus be effectively used in the selective irradiation of a single lobe affected by a disease process. In malignant pulmonary disease the usual route of metastatic processes is by way of the lymphatics. The above-mentioned colloid has been shown to be drained by the lymphatics normally associated with the pulmonary parenchyma, but such drainage requires from ten to fifteen days. The half-life of gold is 2.7 days, and therefore the major part of the radiation has been dissipated by the time the colloid has been deposited in the lymph nodes.[2] It was therefore decided to test some other metallic colloids in order to determine if they might be drained more rapidly. Radioactive silver colloids were prepared as described below and, by means of a bronchoscope, were instilled into single lobes of dogs' lungs. The animals were sacrificed at intervals of two, five, and thirteen days and, as can be seen in Table I, there was quite prompt drainage of an appreciable amount of this material although the individual variation in response was marked.

METHODS

Silver^{111} is produced by an n-γ reaction* on Pd^{110} in a chain-reacting pile. The latter by beta decay becomes Ag^{111} with a half-life of 7.5 days. The silver is separated from the parent palladium as follows: aqua regia dissolves palladium rapidly upon gentle warming (one to two minutes for 1 gram of element) and was thus selected as the solvent. The addition of carrier silver (25 mg. per gram of palladium) to the aqua regia solution results in the precipitation of silver chloride. Adequate mixing of carrier and active silver was obtained by adding concentrated ammonia and stirring until the solution was clear and free from any precipitate. Warming hastens the complexing action of the ammonia (ten to fifteen minutes necessary with a gram of palladium). The silver was then reduced to the metal with ascorbic acid, washed with water and dissolved in dilute nitric acid. For complete removal of chloride and occluded palladium it is desirable to again complex with a small amount of ammonia and reduce. After reduction of silver there is frequently a small quantity (0.1 to 0.5 mg.) of silver metal floating on the supernatant liquid. Since centrifugation (100 ml. centrifuge tubes) and decantation were used throughout, this difficulty was lessened somewhat by the addition of a small amount of alcohol to the solution. The entire procedure requires approximately thirty minutes and at least 90 per cent separation of the

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*Personal communication with S. Allan Lough, Isotopes Division, United States Atomic Energy Commission, Oak Ridge, Tenn.

TABLE I. DISTRIBUTION OF RADIOACTIVE SILVER COLLOIDS FOLLOWING INTRAPULMONARY ADMINISTRATION

DOG	50-12		50-13		51-89*		50-10		51-90*		50-15	
Time after	*2 days*		*2 days*		*2 days*		*5 days*		*5 days*		*13 days*	
	cpm./Gm.	*per cent*	*cpm./Gm.*	*per cent*	*cpm./Gm.*	*per cent*	*cpm./Gm.*	*per cent*	*cpm./Gm.*	*per cent*	*cpm./Gm.*	*per cent*
Treated lobe	125	100	300	100	695,000	100	330	100	320,000	100	149	100
other lobes	102	82	173	58	13	.002	230	70	85		13	9
same lung	4	3	9	3			193	58			18	12
Contralateral	6	5	8	3	48	.006	6	2	170		13	9
lung, various	5	4	6	2			0	0	39		12	8
lobes	0	0	2	0.6			0	0				
Bifurcation node					1,000	0.1			46,000	14		
R. mediastinal node			230	77	269	0.04	5,180	1,500	156,000	49		
L. mediastinal node									91,000	28	72	49
R. tracheal node	0	0			74	0.01	2,190	660	12,000	4		
L. tracheal node					1,470	0.2						
R. bronchial node	190	153	363	120			82	25			254	170
L. bronchial node			9	3			1	0.3			61	41
Inguinal node	0	0										
Axillary node	0	0										
Mesenteric node			4	1			11	3	5		0	0
Liver	0	0	9	3	111	0.01	20	6	660	0.2	7	5
Spleen	0	0	12	4	21	0.003	10	3	224		0	0
Kidney	0	0	18	6			6	2	68		0	0
Total countable radioactivity of instilled silver	44×10^3		48×10^3		28×10^6		30×10^3		28×10^6		40×10^3	

*Ag^{110}, other experiments utilized Ag^{111}.

activity due to Ag^{111} is readily obtained from a single separation. Very small amounts of palladium carried down with silver may readily be removed by repetition of the procedure.

Silver^{110} is produced by an n-γ reaction on silver and therefore is of considerably lower specific activity than the carrier-free material referred to above. It has a half-life of 225 days, and an energetic beta and gamma spectrum. It is dangerous to handle due to its affinity for tissues, and therefore possible carcinogenic action inherent in the long half-life as well as because of its very energetic gamma spectrum.

The silver colloids were made as follows: Silver nitrate containing the radioactive isotope was diluted so as to contain approximately 150 mg. of the element in approximately 20 ml. of water. A few drops of 40 per cent NaOH were added and 1 ml. of 6 per cent gelatin added as a supporting colloid. To this was added 5 ml. of a solution containing 500 mg. of cevitamic acid. The colloid was dispersed immediately and at first appeared black in color but on standing became lighter attaining a yellowish gray appearance to reflected light. It is felt that the change in color is probably due to growth of particle size.

The animals were anesthetized with nembutal and the bronchoscope inserted. A narrow lumen inner tube consisting of a length of thin copper (refrigerator) tubing having the hub of a needle soldered to one end was inserted. The colloid was then instilled by means of a lock type of syringe in a volume of 0.5 ml. per kilogram. Following this about 1 ml. of air was injected in order to empty the contents of the inner tube. The animals were then placed on the treated side until the effects of the anesthetic wore off. The animals were sacrificed at various intervals with an overdose of nembutal. The tissues were removed in order of expected increasing levels of radioactivity, weighed and transferred to metal containers. In samples containing Ag^{111} the tissues were dried at 105° C. overnight and ashed in a muffle furnace at 625° C. Tissues containing Ag^{110} were weighed, transferred, and counted as fresh material.

OBSERVATIONS

The first series of experiments were carried out on four littermate mongrel hound dogs using a silver colloid tagged with Ag^{111}. The silver radioactivities on hand at that time were quite low and therefore the results can be looked on as indicative of lymphatic drainage but not reliable from a quantitative standpoint. In order to check these results two more experiments with two- and five-day intervals between bronchoscopy and sacrifice were carried out using higher levels of radioactivity provided by the Ag^{110} isotope.

In Table I it can be readily seen that there was surprisingly little of the radioactive material found in the contralateral lung, although other lobes than that treated in the same lung showed relatively large amounts of activity in some instances. This was especially true in the earlier experiments involving the animals which received Ag^{111}, and it is thought that perhaps it is referable to unobserved coughing. As in the case of the gold colloids only negligible quantities of the material apparently gained access to the circulation since they would have been rapidly taken up by the liver and spleen by phagocytosis, and these organs showed very low levels of radioactivity. It can be seen that in as little as two days there was a suggestive although not important quantity of the silver taken up by the lymph nodes concerned with pulmonary drainage. However, in five days a marked amount of drainage had occurred suggesting that if therapeutic quantities of the material had been used, considerable irradiation (40 to 50,000 equivalent roentgens) of these lymph nodes could undoubtedly have been attained. At the end of the thirteen-day period it can be seen that the right bronchial node contained considerably higher levels of

radioactivity per gram of tissue than the lobe of the lung originally treated. Wherever taken inguinal, axillary and mesentery nodes showed no activities of consequence.

Post-mortem examination of the animals showed that the lobe into which the silver had been instilled was in every case rubbery in consistency and a brownish black in color. Necrosis with breakdown of the tissue was seen in every instance. In several instances there was evidence of congestion and inflammation of the contralateral lung and in most instances the other lobes of the treated lung showed such changes. It is not possible to say whether the latter pathology is inherent in the use of silver due to the high degree of susceptibility of the dog to pulmonary disturbances.

DISCUSSION

Silver^{111} satisfies most of the criteria which we have arbitrarily set up as satisfactory properties for therapeutic isotopes.[1] Unfortunately, the parent Pd^{110} has an abundance of only 14 per cent and a relatively poor cross section to thermal neutrons. This makes production of the Ag^{111} isotope on a large scale for therapeutic use hardly feasible at the present time. Neither of the above disadvantages are insurmountable, however, since it is possible that should this isotope prove to be of practical value the parent palladium could be obtained in higher concentrations by electromagnetic separation, and the total activities induced increased by utilization of piles with a higher neutron flux.*

In the preliminary experiments it was found that the toxicity of the silver was quite considerable as has been pointed out elsewhere.[4] This objection to the tracer use of Ag^{110} need be of no significance however when one uses the carrier-free Ag^{111}.

It is necessary that the ultimate fate of the silver following its intrapulmonary administration be traced in order to determine that there is no disadvantageous accumulation in any one site of the body and such studies are under way.

In bronchiogenic carcinoma or primary lung tumor the involved lobe of the lung is in any case to be considered expendible. Administration of therapeutic quantities of Au^{198} or the silver isotopic colloids mentioned above result in necrosis and fibrosis of the lung parenchyma into which they have been instilled. In projected treatment of carcinoma of the lung it is anticipated that the silver isotope could be administered for the purpose of ultimate drainage by the lymphatics. Radioactive gold colloids could be used in a similar manner in an attempt to destroy the tumor bed of the lung parenchyma proper. With operable patients in which there is suspected to be some lymphatic metastatic involvement the silver isotope could reasonably be employed, followed in ten days to two weeks by pneumonectomy. In this way irradiation of the lymphatics could be combined with surgery as an approach to treatment of this common malignant condition.

*The silver isotopes used in these experiments were obtained from bombardment in the Oak Ridge Pile.

SUMMARY

Radioactive silver colloids have been administered to dogs by intrapulmonary route and have been shown to be concentrated by the lymphatic system draining the pulmonary region. The possible use of isotopes of this element in the treatment of carcinoma of the lung and bronchiogenic tumors is discussed briefly.

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EFFECTS OF MIXED EXCHANGE RESINS ON ANIONIC AND CATIONIC METABOLISM

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IN A previous publication[1] it was reported that certain resin mixtures incorporating both anion and cation exchangers appeared to be just as efficient in the removal of cations from food in vivo as an equal weight of the carboxylic resin alone. This conclusion was based on evidence from a small number of animals, and only certain specific resin mixtures, but it seemed reasonable on theoretical grounds in that the available capacity of carboxylic resins increases with increasing pH[2]. The presence of the anion exchanger, either Ionac A-300 or Amberlite XE-58 was, however, of very doubtful effect on the metabolism of chlorides; the metabolism of the other anions was not studied. The possibility remained, therefore, that on further study this seeming enhancement of the action of the carboxylic exchanger by the anion exchangers might prove to be illusory. Such mixtures would have therapeutic merit if they removed anions and cations simultaneously, thus achieving a partial demineralization of the food. This demineralization would be of special value in the treatment of the edema of nephritis. In these patients the ability to excrete potassium, as well as sodium, is limited.[3] For this reason it seems contraindicated to attempt to mitigate the acidosis, which results from the ingestion of cation exchange resins,[4] by giving them resin partly in the K-form. The edema of nephritis has been successfully treated by giving only NH_4-form resin, providing that proper measures were taken to prevent acidosis and potassium deficiency.[5]

The purpose of the work reported herein has been to study the principal types of anion exchange resins now available for their effects on anionic metabolism, and for the efficiency of cation removal by simultaneously-fed carboxylic resin. The strongly basic type of resin has not been included in the study because these substances are of quite low capacity.[6] The three basic resins used were as follows: Amberlite XE-58, a weak base; Ionac A-300, an intermediate base;[6] and Duolite A-41, the basic groups of which range from weakly basic tertiary amine to highly basic quaternary amine.[7] A study of the in vitro action of the basic resins has been made, and they have been tested in vivo in conjunction with the carboxylic type exchanger, as represented by Amberlite XE-64 or Duolite CS-101.*

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*Generous supplies of resins were provided through the courtesy of the following: Amberlite XE-58 and XE-64 by Dr. J. C. Winters of Rohm and Haas Co., Philadelphia; Ionac A-300 by Mr. L. W. Winters of American Cyanamid Co., Stamford, Conn., and Duolite A-41 and CS-101 by Dr. I. M. Abrams of Chemical Process Co., Redwood City, Calif.

METHODS OF ANALYSIS

Sodium and potassium were determined in a Perkin-Elmer flame photometer, Model 52A. Calcium was determined by precipitation as the oxalate at pH 5, and subsequent permanganate titration. Magnesium was isolated as the ammonium phosphate salt, as directed by Simonsen, Westover, and Wertman,[8] and the phosphate was estimated by the method of Fiske and Subbarow.[9] Ammonia was determined in urine by the method of Folin and Bell,[10] and titratable acidity was measured by adjusting to pH 7.5 with standard alkali, using glass electrodes for the adjustment. Chlorides were determined in food and feces by the open Carius method,[11] and in urine by the procedure of Schales and Schales.[12] Inorganic phosphate in urine and total phosphorus in food and feces were determined according to Fiske and Subbarow.[9] Conductivity of urine samples was measured by means of a conductivity bridge, and the pH was determined electrometrically.

Samples of food and feces were wet-ashed in sulfuric acid, by adding nitric and perchloric acids. The solution so obtained was used for the determination of sodium, potassium, calcium, magnesium, and phosphate. The last-named was calculated in terms of $HPO^{=}_4$, since in biological systems the tertiary hydrogen is usually not replaced. The composition of the food, per 90 Gm. basal diet (or 100 Gm. resin-containing diet), was as follows, as meq.: sodium, 28; potassium, 17; calcium, 24; magnesium, 10; $HPO^{=}_4$, 31; chloride, 29.

IN VITRO EXPERIMENTS

Phosphate solutions of pH 6.4 and 7.4 were prepared. Solution I (pH 6.4) had the composition previously described;[13] Solution II contained, in 5,000 ml., the following: KH_2PO_4, 3.7 Gm.; K_2HPO_4, 19.8 Gm.; $NaH_2PO_4.H_2O$, 11.2 Gm.; $Na_2HPO_4.12H_2O$, 49.2 Gm. These solutions analyzed, respectively, 275 and 203 meq. of $HPO^{=}_4$ per liter. The resins* to be tested were shaken with these solutions for three hours, the proportion being 16 Gm. per liter, then the suspensions were filtered and the filtrates were analyzed for phosphate. From the net change in composition the phosphate uptake of the resin was calculated; the results are given in Table I.

As revealed by these experiments, there seems to be no important difference between Amberlite XE-58 and Ionac A-300 except that at both initial pH values, in proportion to its total capacity, the Ionac A-300 appears to have a greater uptake. In this sense it gives some evidence of being a stronger base than the Amberlite XE-58. Both resins remove

TABLE I. PHOSPHATE UPTAKE* OF SOME ANION EXCHANGE RESINS IN VITRO

RESIN	SOLUTION I		SOLUTION II	
	UPTAKE	FINAL pH	UPTAKE	FINAL pH
Amberlite XE-58†	3.6	7.1	0.8	8.3
Ionac A-300†	3.6	7.2	0.8	8.7
Duolite A-41‡	2.7	7.1	1.2	9.9
Carbo-Resin§	0.6	6.4	0.0	6.9

*As meq. of $HPO^{=}_4$ per Gm.

†The total capacities of these resins are given as 10.0, and 7.3 meq. per gram, respectively.

‡The total capacity of this resin; as determined by its uptake of HCl from 0.1N HCl, is 5 meq. per gram.

§Product of the Eli Lilly Co., Indianapolis, Ind. This is stated to be a mixture of Amberlite XE-96 (carboxylic), partly in the K-form, and Amberlite XE-58[14].

markedly less phosphate from Solution II than from Solution I. This is to be expected, since the capacity of the weakly basic type of exchanger decreases with increasing pH. The Duolite A-41 is of lower capacity than either of the other resins, but it seems definitely to be a stronger base in that its phosphate uptake is greater at the higher initial pH, and it forces the final pH to a much higher level. One brand of sodium-removing resins[14] con

*In the OH-cycle, 40-mesh.

tains about 12 per cent of basic resin. On this basis, its phosphate uptake at pH 6.4 exceeds slightly what would be expected from its content of Amberlite XE-58 alone, perhaps because of the simultaneous acidifying action of the carboxylic resin.

IN VIVO EXPERIMENTS

The methods used in conducting the animal experiments have been described previously in some detail;[13] identical procedures were used in this work. The resin mixtures were prepared so that the entire range of proportions of anion and cation exchangers was covered; i.e., the amount of carboxylic resin ranged from 0 to 10 Gm. per 100 Gm. of diet, as the amount of basic resin ranged from 10 to 0 Gm. The carboxylic resins were always

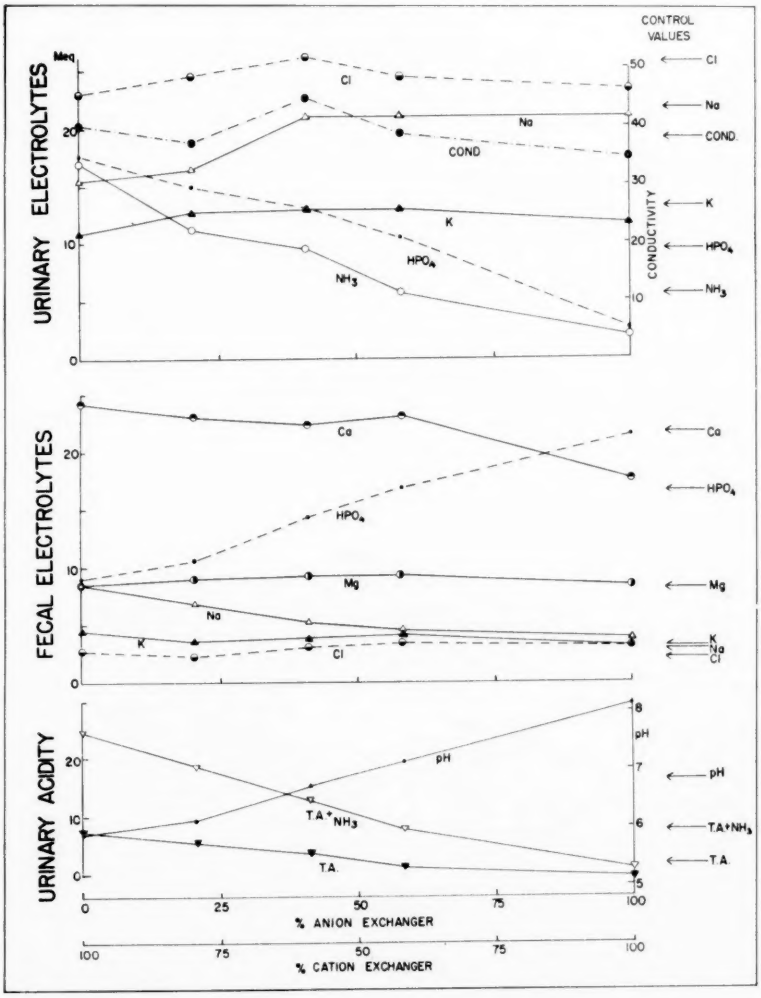

Fig. 1.—Effect of ingesting Amberlite XE-64 (carboxylic) and/or Amberlite XE-58 (weak base) on the mineral metabolism and neutrality regulation mechanisms of rats. The total resin content of the diet was 10 per cent, and all values are plotted in terms of meq. per 90 Gm. basal diet (or 100 Gm. of resin-containing diet) consumed. Conductivity is also given in these terms, using a solution of C. P. sodium chloride as a standard.

fed in the H-cycle. The Amberlite XE-64 contained 10 per cent of moisture as fed, but the other resins were essentially moisture-free as supplied, or had been dried at 75° C. overnight before using. All samples were ground to pass through a 40-mesh screen.

Three metabolic experiments were conducted in all; Fig. 1* shows the results of feeding mixed Amberlites XE-64 and XE-58, Fig. 2 shows the results of feeding mixed Amberlite XE-64 and Ionac A-300, and Fig. 3 shows the results of feeding mixed Duolites CS-101 and A-41.

DISCUSSION

The physiologic action of the separate and mixed ion exchange resins, as shown in Figs. 1 to 3, reveals some interesting features. There are marked

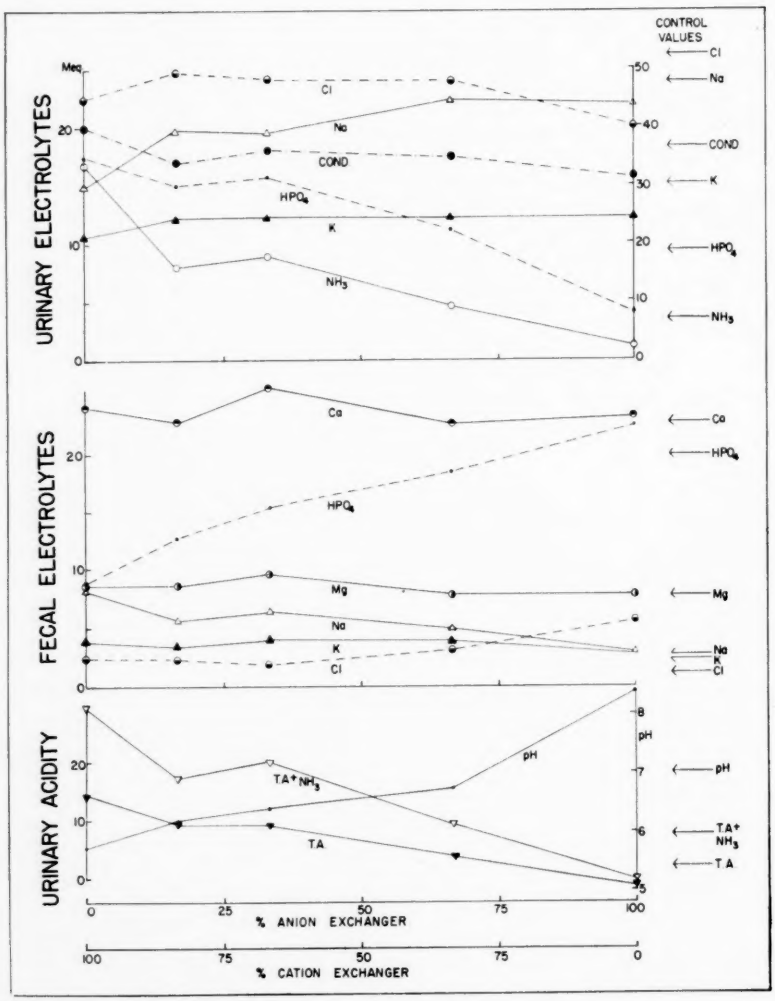

Fig. 2.—Effect of ingesting Amberlite XE-64 (carboxylic) and/or Ionac A-300 (intermediate base) on the mineral metabolism and neutrality regulation mechanisms of rats. The values are plotted in the same manner as in Fig. 1.

*The author is indebted to Mrs. Edith Froelich for preparing these illustrations.

similarities in the action of the three basic resins, and some differences. In all cases, for example, as the amount of basic resin increases, there is an increase of urinary pH, sodium, and potassium. Concomitantly, there is a decrease in urinary ammonia, titratable acidity, and phosphate. In the feces the changes, where the same constituents were determined, are naturally in the opposite direction. The effects on sodium and potassium metabolism are about as would be predicted from the combinations fed; i.e., the fecal excretion decreases as the amount of carboxylic resin decreases. The interesting point is that there is no evidence that any of the three basic resins caused a sufficient local increase of pH in the intestinal fluids to potentiate the action of the carboxylic resins. Even in the presence of the relatively strong base, Duolite A-41 (Fig. 3), the

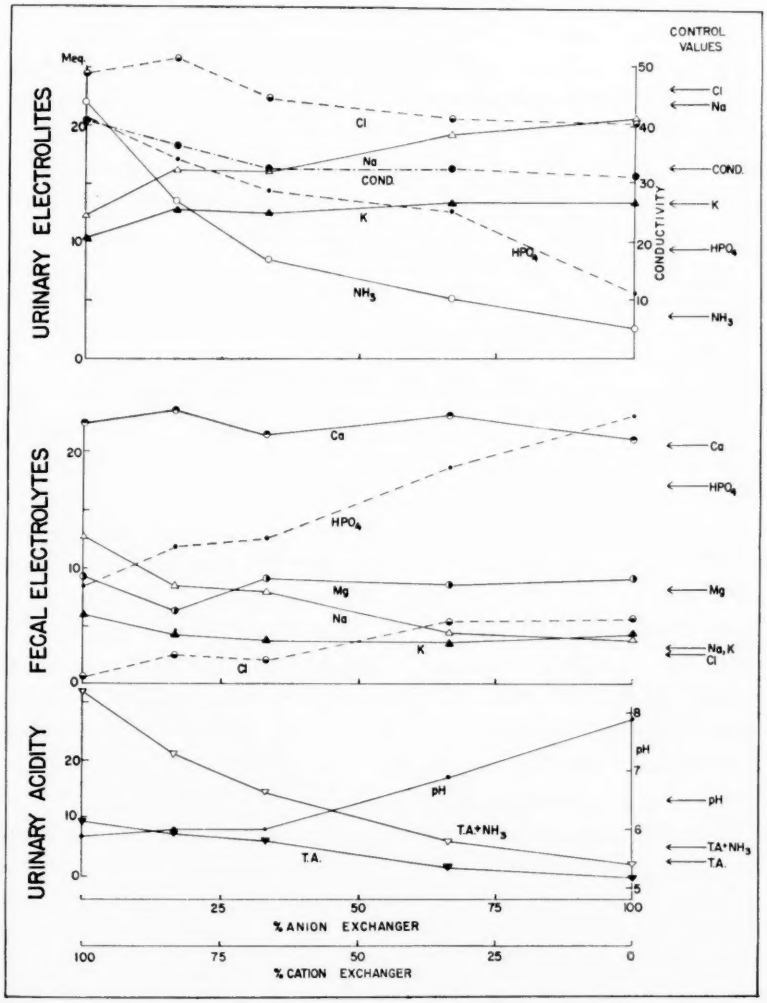

Fig. 3.—Effect of ingesting Duolite CS-101 (carboxylic) and/or Duolite A-41 (containing both weakly and strongly basic groups) on the mineral metabolism and neutrality regulation mechanisms of rats. The values are plotted in the same manner as in Figs. 1 and 2.

extra fecal sodium falls off in almost direct proportion to the amount of Duolite CS-101 in the diet. The action of the latter resin alone is quite satisfactory, since its apparent cation uptake in the intestine is about 1.0 meq. sodium, 0.3 meq. potassium, 0.2 meq. calcium, and 0.1 meq. magnesium, per gram. The excess acid production (i.e., urinary titratable acidity plus ammonia, for the 100 per cent CS-101 animals, less that of the controls), however, is nearly 3 meq. per gram. This would indicate an actual cation uptake of 3 meq. per gram, rather than the 1.6 meq. total found for the four ions named above.*

The total electrolyte content of the urine, as indicated by its conductivity, is not significantly altered by the various resin combinations, except that it is slightly higher on the diets containing the highest proportions of Duolite CS-101. This effect seems related to the intense acidifying action of the resin (due to the fecal losses of cations), resulting in high outputs of ammonia and phosphate. If mixtures of anion and cation exchange resins really constituted an effective means of demineralizing ingested food, the conductivity curves would be of the catenary type.†

Perhaps the most interesting effect noted, in all of the resin combinations, is on the metabolism of phosphate. The carboxylic resins greatly increase the urinary, and reduce the fecal, output, while the basic resins cause the opposite changes to occur. These changes appear to have two possible explanations: (1) the resin may react with calcium which would otherwise be excreted as the phosphate, thereby releasing phosphate for absorption, or (2) the change may be mediated by the kidney as a means of maintaining acid-base balance. The explanation favored by the author is that the carboxylic resin increases phosphate absorption by effecting a decomposition of calcium phosphate, while the basic resins bind phosphate; the various resin mixtures would naturally give intermediate results. The data show how it is possible for the metabolism of an ion to be radically altered by the ingestion of a resin carrying the same charge as the ion itself. The amount of phosphate bound by the resins in vivo is in no case large; i.e., it is from 0.3 to 0.6 meq. per Gm., but the claim[15] that feeding Amberlite XE-58 results in no fecal loss of phosphate is not substantiated.

As regards chloride metabolism, it seems clear that Duolite A-41 increases the fecal output (by about 0.3 meq. per Gm.), and that Amberlite XE-58 does not. This substantiates the claim[15] made for the latter. The effect of Ionac A-300 is somewhat doubtful, for reasons to be pointed out below.

In general, the (Amberlite XE-64 plus Ionac A-300) mixtures behaved the same as the other two. There seems to be some binding of phosphate and chloride at the higher levels of A-300, but it should be pointed out that the fecal sodium and potassium in these animals also remained above control levels. This provides an example of an increase in the fecal output of cations, caused by the ingestion of a basic resin. The explanation seems to lie in the fact that

*It is not necessary that these two figures should agree. See below.

†It must be recalled, however, that conductivity varies with the individual ions, as well as the total number present. The substitution of ammonium and hydrogen ions for sodium ions tends to increase conductivity, and increasing phosphate output has the same effect.

the feces obtained from these animals were very bulky, and the relatively large amount of water retained in the intestine resulted in some retention of electrolytes also. The retention of chloride may, therefore, not represent an actual binding. The low T.A. + NH_3 in the pair receiving ⅙ Ionac A-300 and ⅚ Amberlite XE-64 (Fig. 2) reflects the low fecal output of cations. This is undoubtedly an effect which would not always be observed, but chanced to occur here.

Calcium and magnesium absorption were not drastically altered by the resins, at least so far as the net result was concerned. In general, the fecal outputs of these elements were slightly increased when a carboxylic resin was present in the diet.

SUMMARY

The action of carboxylic exchange resin and several types of basic resins, both singly and in combination, has been studied in vivo. The total urinary electrolyte output remains essentially unchanged regardless of the resin combination fed; there is no evidence that the basic resins are capable of potentiating in any manner the action of the carboxylic resin. In all cases studied, the combination of approximately 65 per cent anion exchanger, 35 per cent cation exchanger is physiologically neutral; i.e., the urinary output of acid is the same as that of the controls. The most striking effect observed is on phosphate metabolism: the cation exchanger increases phosphate absorption, while the anion exchangers decrease it. Chloride metabolism is only slightly altered by the basic resins and, in some cases, the effect may be attributed to an abnormal retention of water in the intestine. Since the addition of basic exchangers to the carboxylic resin reduces its capacity for sodium removal, the use of mixed anion-cation exchangers in edema appears to offer no clear-cut advantage.

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CROSS-RESISTANCE TO ANTIBIOTICS

Effect of Repeated Exposures of Bacteria to Streptomycin or Neomycin on the Resistance to Both of These and to Six Other Antibiotics

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In A recent study[1] on the cross-resistance resulting from repeated exposures of a number of bacterial strains to aureomycin, terramycin, chloramphenicol, and neomycin, it was shown that (1) mutual cross-resistance between aureomycin and terramycin developed most regularly and clearly; (2) cross-resistance between chloramphenicol and either aureomycin or terramycin developed in some organisms but not in others, and (3) no cross-resistance developed between neomycin and any one of the other three antibiotics in any of the organisms tested. At the time when that study was completed there had been some reports which indicated that neomycin was active against streptomycin-resistant organisms, but there was little information to indicate whether or not organisms which had acquired resistance to neomycin as a result of exposures to that agent would also become resistant to streptomycin. When such organisms were tested, it was found that streptomycin-resistance did indeed increase significantly in all but one of the strains that had developed increased resistance to neomycin. This result was not anticipated, and it was felt that the problem of the cross-resistance between neomycin and streptomycin deserved further exploration.

The present study is concerned with attempts to increase the resistance of several organisms to streptomycin and neomycin by repeated subcultures in the presence of progressively increasing concentrations of each of these antibiotics. After a number of such subcultures, the organisms and their parent strains, which had been subcultured in parallel using the same media but without antibiotics, were then tested for sensitivity to each of these two antibiotics and to a number of others. In the course of this study a streptomycin-dependent variant of one of the organisms developed. The effect of neomycin on the growth of this variant was also tested.

MATERIALS AND METHODS

Organisms.—The strains used in this study, their designations and their sources were as follows:

Ec, a mucoid strain of *Escherichia coli* from urine of a patient with pyelonephritis;

Em, the McLeod strain of *Escherichia coli,* which is not mucoid;

Kp, a strain of *Klebsiella pneumoniae,* type A from sputum of a patient with pneumonia;

Sh, a strain of *Streptococcus pyogenes* from the throat of a patient with scarlet fever;

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C203, a subculture of this strain of *Streptococcus pyogenes*, Group A, type M3 obtained from Dr. E. L. Updyke, Communicable Disease Center, U. S. Public Health Service, Chamblee, Ga.;

Sv, a strain of *Streptococcus mitis* from the blood of a patient with subacute bacterial endocarditis; and

Pn3, a strain of *Diplococcus pneumoniae*, type 3, from sputum of a patient with lobar pneumonia. These strains, with the exception of Em and C203 had recently been isolated in this laboratory; none of them had had any known previous exposure to any antibiotics.

Media.—Heart infusion agar (Difco) and brain-heart infusion broth, both at pH ± 7.4, were used throughout and are referred to as agar and broth, respectively. All dilutions of antibiotics and of cultures were made in broth. Defibrinated equine blood was incorporated in the culture media, 10 per cent in agar and 0.5 per cent (final) in broth, to enhance the growth of the gram-positive organisms and to serve as an indicator of growth and hemolysis.

Antibiotics.—Neomycin sulfate was supplied by Dr. E. L. Burbidge of the Upjohn Co. in sterile vials, each containing 0.5 Gm. Streptomycin was obtained commercially as the calcium chloride complex. Stock solutions of these two agents were prepared in adequate quantities for the entire study; they were distributed in separate tubes, each with enough for one subculture. The tubes were then sealed and stored at −25° C. (in an electric deep-freeze) and individual tubes were thawed, opened, and used at the time of each subculture. Potassium penicillin G was obtained commercially; aureomycin hydrochloride was supplied in crystalline form in sterile vials by Dr. Stanton M. Hardy of the Lederle Laboratories; terramycin hydrochloride in similar form was furnished by Dr. Gladys L. Hobby of Chas. Pfizer & Co.; chloramphenicol (Chloromycetin) was provided by Dr. E. A. Sharp of Parke-Davis & Co.; polymyxin B (Aerosporin) was supplied by Dr. Donald S. Searle of Burroughs Wellcome Co., and bacitracin was obtained from Dr. Lawrence W. Smith of Commercial Solvents Corp. Solutions of the last six antibiotics were freshly prepared at the time they were used.

Tests for Sensitivity.—In experiments employing subcultures in broth, the tube-dilution method of Rammelkamp and Maxon[2] was used with minor changes. Serial twofold dilutions of antibiotic in a suitable range were distributed in 0.5 ml. amounts in a series of 13 by 100 mm. tubes. To each was added 0.5 ml. of a 10^{-4} dilution of a fully grown culture in broth containing 1 per cent defibrinated equine blood. The number of viable organisms in the inoculum was estimated each time by counting the number of colonies which developed in an agar pour plate containing 1 ml. of a 10^{-7} dilution of the culture used. The tubes were then incubated at 37° C. for eighteen to twenty-four hours, after which the appearance of growth, as indicated by turbidity and/or hemolysis or discoloration of the blood, was noted. The sensitivity of any strain was defined as the minimum concentration of antibiotic which completely inhibited growth as indicated by the absence of turbidity or hemolysis. The method used in the studies employing solid media was that described by Gocke and Finland,[1] except that the readings were made after incubation for twenty-four hours. A standard stock strain (*K. pneumoniae*, strain T) was included with each test as a control on the potency of the antibiotics.

Development of Resistant Strains.—Serial subcultures were made daily in graded concentrations of antibiotic, both in broth and on agar. Each subculture was made essentially as in the tests for sensitivity, the inoculum being obtained from the tube or plate containing the maximum concentration of antibiotic which yielded nearly optimum (++ or better) growth. When it was necessary to skip a day the cultures were stored in a refrigerator at 5° C. during that day. Subcultures were also made each time with antibiotic-free media in an attempt to recognize any antibiotic-dependent variants which might arise.

RESULTS

Development of Cross Resistance in Broth Cultures.—In an initial study, five strains of different organisms were used. Each strain was transferred in

TABLE I. CHANGES IN RESISTANCE OF FIVE BACTERIAL STRAINS TO STREPTOMYCIN AND NEOMYCIN AFTER REPEATED SUBCULTURES IN ANTIBIOTIC-FREE BROTH AND IN BROTH CONTAINING INCREASING CONCENTRATIONS OF ONE OR THE OTHER OF THESE ANTIBIOTICS

STRAIN	INITIAL SENSITIVITY* μG/ML.	SENSITIVITY* AFTER 35 SUBCULTURES IN BROTH CONTAINING:					
		NO ANTIBIOTIC		STREPTOMYCIN		NEOMYCIN	
		μG/ML.	CHANGE†	μG/ML.	CHANGE†	μG/ML.	CHANGE†
Sensitivity to Streptomycin							
Ec	1.6	3.1	2	>12,800	> 8,192	200	128
Kp	0.4	0.8	2	>12,800	>32,768	100	256
Sh	6.3	12.5	2	12.5	2	25	4
Sv	6.3	12.5	2	12,800	2,048	1,600	256
Pn3	25.0	25.0	1	12,800	512	25	1
Sensitivity to Neomycin							
Ec	1.6	1.6	1	12.5	8	200	128
Kp	0.4	0.4	1	S-D‡	S-D‡	100	256
Sh	25.0	25.0	1	12.5	½	25	1
Sv	25.0	50.0	2	100.0	4	400	16
Pn3	100.0	100.0	1	100.0	1	200	2

*Minimum complete inhibiting concentration.
†Fold increase from initial sensitivity.
‡Organisms became streptomycin-dependent (See Table IV).

three parallel series in broth containing (1) no antibiotic, (2) streptomycin, and (3) neomycin. The thirty-fifth transfer of each series was tested for sensitivity to both antibiotics. The results are shown in Table I.

The sensitivity of each of the five organisms to both antibiotics was the same, within the error of the test, before and after the thirty-five subcultures in antibiotic-free broth. After thirty-five subcultures in broth containing streptomycin, four of the five strains showed a marked increase in resistance to the homologous antibiotic but *Sh*, the hemolytic streptococcus, retained its original sensitivity. The strain of *K. pneumoniae* failed to grow well in the absence of streptomycin, that is, it had become streptomycin-dependent. Further studies with this variant are described below. When tested for sensitivity to neomycin, two of the strains which had become markedly resistant to streptomycin showed only a slight increase in resistance to neomycin, while the hemolytic streptococcus and the pneumococcus retained their original sensitivity to the latter agent.

Following the thirty-five subcultures in broth containing neomycin, three of the five strains increased significantly in resistance to the homologous antibiotic and each of these three strains showed a similar or greater increase in resistance to streptomycin. The hemolytic streptococcus and the pneumococcus did not show any significant change in resistance to either agent as a result of the repeated exposures to neomycin.

Comparison of Broth and Agar.—Murray and associates[3] have shown that streptomycin resistance develops more rapidly when organisms are subcultured on streptomycin agar than when they are grown in streptomycin broth. It was of interest to see whether the development of neomycin resistance occurred more rapidly on agar than in broth containing this antibiotic. The study of cross-resistance was therefore repeated, this time carrying out six parallel series of daily transplants with each of five strains; three of the subcultures were made,

as before, in broth containing streptomycin, neomycin and no antibiotic and the other three were done on agar containing streptomycin, neomycin and no antibiotic. Three of the five strains were the same as those used in the first experiment, but other strains of *E. coli* and hemolytic streptococcus were chosen for this experiment. A total of twenty-seven such parallel subcultures were carried out. The changes in sensitivity to the homologous antibiotic of each of the successive subcultures are shown graphically in Fig. 1.

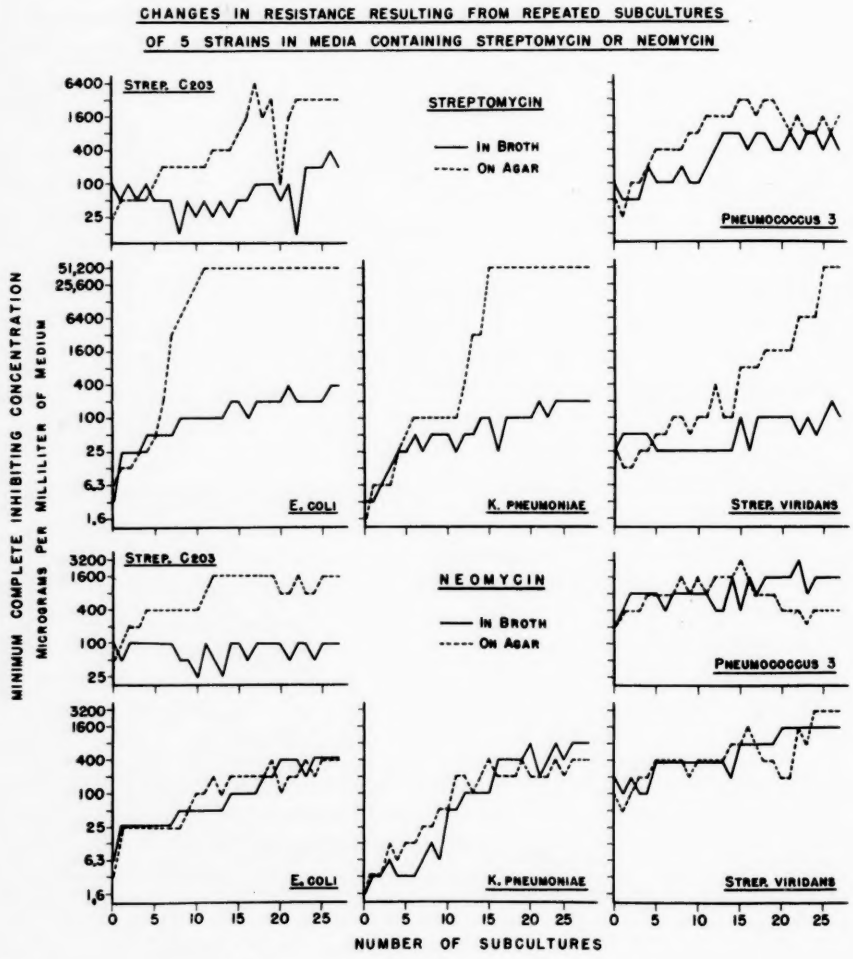

Fig. 1.

It is seen that streptomycin resistance developed more rapidly and, in four of the five strains also to a much higher degree when the subcultures were made on the streptomycin-agar. There was no difference in the neomycin resistance resulting from subcultures on agar or in broth containing that antibiotic, except possibly in the C203 strain of hemolytic streptococcus.

In this connection it is of interest to compare the results obtained in broth in the two experiments. Three of the strains (*Kp*, *Sv*, and *Pn3*) were the same in both experiments except that they had undergone additional transfers in antibiotic-free broth before being used in the second experiment. The most striking difference was in the degree of streptomycin resistance acquired as a result of the transfers in streptomycin broth. The larger number of transfers in the first experiment resulted in a much greater increase in streptomycin resistance, the differences being 200- to 1600-fold. In the first experiment the increase in streptomycin resistance of the same three strains between the twenty-seventh and the thirty-fifth subcultures in streptomycin broth was 32- to 128-fold. Differences of similar magnitude were noted in the case of the strains of *E. coli* used in the two experiments, but neither of the two strains of *Str. pyogenes* showed any marked increase in streptomycin resistance in the broth subcultures.

There was no significant difference in the degree of neomycin resistance resulting from the subcultures in neomycin broth in the two experiments. However, there was a considerably greater increase in the cross resistance to streptomycin resulting from the larger number of subcultures in neomycin broth, particularly in strains *Kp* and *Sv*.

Cross-Resistance Between Streptomycin and Neomycin Following Repeated Exposures of Organisms to One of These Agents in Broth or on Agar.—The twenty-seventh subcultures of each of the six series were all tested for sensitivity to streptomycin and neomycin. The strains of the three series that had been carried in broth were tested by the tube-dilution method and those which had been carried on agar were tested by the plate-dilution method. The results are shown in Table II.

It is seen, first of all, that the sensitivity of each of the five organisms to both streptomycin and neomycin was the same before and after the twenty-seven subcultures in broth or on agar which contained no antibiotic. After the subcultures in broth and on agar containing streptomycin, all five strains increased in resistance to that antibiotic; however, a much greater degree of resistance developed when these transfers were made on agar. Resistance to neomycin increased significantly in four of the five strains that had been exposed to streptomycin in broth, but only two of the strains which had become streptomycin-resistant as a result of subcultures on streptomycin-agar increased appreciably in resistance to neomycin.

Following the twenty-seven subcultures in the presence of neomycin, all but one of the strains increased in resistance to the homologous antibiotic in each of the series; the pneumococcus failed to increase in resistance to neomycin after it was subcultured on agar and C203 did not become more resistant after the twenty-seven transfers in broth containing that antibiotic. Cross-resistance of a considerable degree developed to streptomycin in three of the strains of the neomycin agar series and in two of those in the neomycin broth series. The pneumococcus, which was not significantly increased in resistance to neomycin after the twenty-seven subcultures on agar containing that antibiotic, had nevertheless increased appreciably in resistance to streptomycin as a result of

TABLE II. CHANGES IN RESISTANCE OF FIVE BACTERIAL STRAINS TO STREPTOMYCIN AND NEOMYCIN AFTER REPEATED SUBCULTURES IN BROTH OR ON AGAR WITH OR WITHOUT ONE OF THESE ANTIBIOTICS

STRAIN	INITIAL SENSITIVITY*	SENSITIVITY* AFTER 27 SUBCULTURES:					
		WITHOUT ANTIBIOTIC		WITH STREPTOMYCIN IN MEDIUM		WITH NEOMYCIN IN MEDIUM	
	μG./ML.	μG/ML.	CHANGE†	μG/ML.	CHANGE	μG/ML.	CHANGE
		Sensitivity to Streptomycin on Agar‡					
Em	6.3	6.3	1	>51,200	>8,192	200	32
Kp	1.6	1.6	1	>51,200	>32,768	100	64
C203	50	25	½	3,200	64	100	2
Sv	25	25	1	51,200	2,048	100	4
Pn3	25	25	1	3,200	128	1,600	64
		Sensitivity to Streptomycin in Broth					
Em	6.3	6.3	1	400	64	200	32
Kp	3.1	3.1	1	200	64	50	16
C203	50	25	½	400	8	50	1
Sv	25	25	1	200	8	50	2
Pn3	50	50	1	800	16	50	1
		Sensitivity to Neomycin on Agar					
Em	1.6	3.1	2	12.5	8	400	256
Kp	1.6	3.1	2	1.6	1	400	256
C203	100	50	½	100	1	1,600	16
Sv	100	100	1	100	1	3,200	32
Pn3	200	200	1	800	4	400	2
		Sensitivity to Neomycin in Broth					
Em	6.3	3.1	½	100	16	400	64
Kp	1.6	0.8	½	200	128	1,600	1,024
C203	100	100	1	200	2	100	1
Sv	200	100	½	800	4	1,600	8
Pn3	200	200	1	1,600	8	1,600	8

*Minimum complete inhibiting concentration.
†Fold increase over initial sensitivity.
‡The type of medium used in the tests corresponded to that used for the subcultures.

these transfers. It is interesting to note, however, that this strain had been somewhat more resistant to neomycin in some of the earlier transfers, as shown in Fig. 1.

Effect of Repeated Exposures to Streptomycin or Neomycin on the Sensitivity to Six Different Antibiotics.—Each of the strains of the six series, after they had been transferred twenty-seven times in broth or on agar containing either streptomycin, neomycin, or no antibiotic, were also tested for sensitivity to penicillin, aureomycin, terramycin, chloramphenicol, bacitracin, and polymyxin B. The strains which had been transferred on agar were tested by the plate dilution method and those which had been subcultured in broth were tested by the tube dilution method. The results are shown in Table III. When the strains which had been subcultured in broth were tested by the plate dilution method, the results were essentially the same, so these were omitted from the table.

For the most part, the sensitivity of each strain to each of the six antibiotics to which they had not been exposed was the same after the twenty-seven transfers in the presence of streptomycin or neomycin as they were after the same number of subcultures on similar antibiotic-free media. However, in several instances (almost one-third of the tests) there was an apparent increase in

TABLE III. EFFECT OF TWENTY-SEVEN TRANSFERS IN MEDIA CONTAINING STREPTOMYCIN, NEOMYCIN, OR NO ANTIBIOTIC ON THE SENSITIVITY OF FIVE ORGANISMS TO PENICILLIN, AUREOMYCIN, TERRAMYCIN, CHLORAMPHENICOL, BACITRACIN, AND POLYMYXIN B

	MINIMUM COMPLETE INHIBITING CONCENTRATION, MICROGRAMS PER MILLILITER											
	PENICILLIN		AUREOMYCIN		TERRAMYCIN		CHLORAMPHENICOL		BACITRACIN		POLYMYXIN B	
STRAIN*	BROTH†	AGAR†	BROTH	AGAR	BROTH	AGAR	BROTH	AGAR	BROTH	AGAR	BROTH	AGAR
Em-O	6.3	100	1.6	6.3	1.6	12.5	1.6	12.5	50	100	0.4	1.6
Em-S	12.5	100	3.1	6.3	3.1	3.1	1.6	3.1	200	100	0.4	0.8
Em-N	6.3	100	6.3	6.3	1.6	3.1	1.6	3.1	100	100	0.4	0.4
Kp-O	12.5	100	3.1	6.3	3.1	6.3	1.6	12.5	200	400	0.8	1.6
Kp-S	6.3	12.5	3.1	3.1	1.6	1.6	1.6	1.6	200	100	0.4	0.4
Kp-N	6.3	25	1.6	6.3	3.1	3.1	0.8	6.3	200	400	0.4	0.8
C203-O	0.01	0.8	0.4	6.3	3.1	12.5	3.1	6.3	0.01	0.2	50	>400
C203-S	0.01	0.01	0.4	0.8	1.6	0.8	1.6	3.1	0.01	0.01	50	200
C203-N	0.01	0.01	0.4	3.1	3.1	3.1	3.1	6.3	0.01	0.01	50	200
Sv-O	0.02	0.1	0.4	1.6	6.3	1.6	3.1	25	0.04	0.4	100	>400
Sv-S	0.02	0.02	0.4	0.8	6.3	1.6	3.1	3.1	0.04	0.8	100	>400
Sv-N	0.01	0.01	0.8	3.1	6.3	0.8	3.1	1.6	0.02	0.01	50	200
Pn3-O	0.02	0.02	0.2	0.8	3.1	0.4	3.1	3.1	0.8	0.8	50	>400
Pn3-S	0.01	0.02	0.2	0.8	3.1	0.8	1.6	3.1	1.6	0.8	50	400
Pn3-N	0.01	0.01	0.2	6.3	1.6	0.8	1.6	6.3	0.01	3.1	50	>400

*Em = McLeod strain of *E. coli*; Kp = *K. pneumoniae*, type A; C203 = Group A, type M3 strain of *Strep. pyogenes*; Sv = *Strep. mitis* and Pn3 = type 3 pneumococcus. O, S and N indicate that the organism had been transferred 27 times in media containing no antibiotic, streptomycin or neomycin, respectively.

†Broth indicates that the twenty-seven transfers were made and the tests for sensitivity were carried out in fluid media; agar indicates that the twenty-seven transfers and the tests for sensitivity were all done on solid media.

sensitivity (fourfold or greater) to some of these other antibiotics following the exposure of the strains to streptomycin or neomycin on agar; the same, however, was observed only once after the subcultures in broth. With only 2 possible exceptions, no increase in resistance occurred to any of the 6 antibiotics to which the strains had not previously been exposed.

Development of a Streptomycin-Dependent Variant.—Incidental to the initial study, the cultures used for the inocula in the successive transfers in broth were streaked each time on the surface of antibiotic-free agar and on agar containing an arbitrary concentration of the same antibiotic in which it was being subcultured. Following the thirty-fifth subculture in streptomycin, it was noted that the strain of *K. pneumoniae*, which had previously grown well on a plain agar plate and on streptomycin agar, failed to grow on the antibiotic-free agar, but grew luxuriantly on agar containing 320 μg of streptomycin per milliliter. The other strains of both the streptomycin and the neomycin series all grew well under similar circumstances both on antibiotic-free agar and on agar containing the antibiotic used in the transfer media. Successive subcultures of this strain of *K. pneumoniae* on agar with and without streptomycin indicated clearly that a streptomycin-dependent variant had emerged[4] which grew well in media containing a wide range of concentrations of streptomycin. By streak plate and pour plate cultures in graded concentration of streptomycin, the optimum growth of this strain was shown to occur in media which contained between 400 and 3200 μg of streptomycin per milliliter.

Effect of Neomycin on the Growth of the Streptomycin-Dependent Strain of K. pneumoniae.—Two experiments were carried out in order to determine whether neomycin would support the growth of the streptomycin-dependent variant either alone or in the presence of suboptimal concentrations of streptomycin. The dependent strain was maintained by serial transfers both on agar and in broth, each containing 800 μg of streptomycin per milliliter. The organism was checked each time for dependence by subcultures to both antibiotic-free and streptomycin-containing agar and broth. Its inability to grow without streptomycin verified its dependence. A single colony was then picked from a pour plate in agar containing 800 μg per milliliter of streptomycin and this was used after it was again demonstrated to be streptomycin-dependent.

Agar plates containing varying amounts of streptomycin with and without graded concentrations of neomycin were then prepared. The culture derived from the single colony and grown in broth containing 800 μg per milliliter of streptomycin was then washed and resuspended in antibiotic-free broth and a 2 mm. loopful of this suspension was streaked on each of the prepared plates and on a plain agar plate. The growth observed after incubation for forty-eight hours is shown in the upper portion of Table IV.

It is seen that the streptomycin-dependent organism grew in the absence of neomycin but in the presence of as little as 6.3 μg of streptomycin per milliliter. No growth occurred on the antibiotic-free agar nor on any of the plates which contained neomycin but no streptomycin. Not only did neomycin not support the growth of the organism in the absence of streptomycin, but in concentrations

of 0.8 μg or more per milliliter the neomycin partially inhibited its growth, and in concentrations of 25 μg per milliliter or more of neomycin the organisms failed to grow even in the presence of high concentrations of streptomycin.

A culture similar to that used in the streak plates was also used for quantitative studies. For this purpose, the washed and resuspended culture of the streptomycin-dependent variant was diluted to 10^{-7} in antibiotic-free broth and 1 ml. of this dilution was incorporated in agar pour plates with various concentrations of streptomycin and/or neomycin. The number of colonies were counted after incubation for forty-eight hours. The results are shown in the lower part of Table IV and are essentially similar to the qualitative findings just presented. The neomycin could not replace the streptomycin in supporting the growth of this streptomycin-dependent strain, nor did it enhance its growth in the presence of suboptimal concentrations of streptomycin.

TABLE IV. EFFECT OF VARIOUS CONCENTRATIONS OF STREPTOMYCIN AND NEOMYCIN ON THE GROWTH OF A STREPTOMYCIN-DEPENDENT VARIANT OF KLEBSIELLA PNEUMONIAE

CONCENTRATION OF NEOMYCIN μG PER ML.	CONCENTRATION OF STREPTOMYCIN, μG PER ML.							
	3200	1600	800	400	100	25	6.3	0
	*Growth on Agar Streak Plates**							
50	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0
12.5	±	±	±	0	0	0	0	0
6.3	+	±	±	±	±	0	0	0
3.1	++	++	+	+	+	±	±	0
1.6	+++	+++	++	++	++	±	±	0
0.8	+++	+++	+++	+++	+++	++	±	0
0.4	+++	+++	+++	+++	+++	++	+	0
0.2	+++	+++	+++	+++	+++	++	+	0
0	+++	+++	+++	+++	+++	++	+	0
	*Number of Colonies in Agar Containing 1 ml. of 10^{-7} Dilution of Culture**							
25	0	0	0	0	0	1	0	0
6.3	32	34	36	18	0	0	1	0
0.4	112	98	134	127	80	3	0	0
0	96	126	152	132	13	0	0	0

*The inoculum was obtained from a fully grown culture in broth containing streptomycin 800 μg per milliliter which, in turn, was derived from a single colony picked from a pour-plate of agar containing the same concentration of streptomycin; the culture was washed and resuspended in antibiotic-free broth. The streak-plates were inoculated directly from the resuspended culture with a 2 mm. loop; the pour plates were made from the culture after dilution in antibiotic-free broth. Readings of growth and colony counts were made after incubation for forty-eight hours.

*Identity of the Strains.**—Because of the nature of the manipulations involved in this study, the possibilities for inadvertent errors were constantly kept in mind. All doubtful or contaminated cultures were promptly discarded; for this purpose colonial morphology proved to be the best guide. In addition, at the conclusion of the experiment the final subcultures in each series were studied simultaneously for their serologic and/or biochemical reactions as a further check on the identity of the strains and also to determine whether the exposures to the antibiotics had altered these basic characteristics. The results may be summarized briefly for each of the organisms.

*We are indebted to Marion E. Lamb of the Mallory Institute of Pathology and Dr. E. L. Updyke of the Communicable Disease Center, U. S. Public Health Service, for the identification of the strains.

E. coli.—Both strains, *Ec* and *Em*, retained their original colonial and microscopic morphology and biochemical reactions after the subcultures with or without streptomycin or neomycin. They all fermented lactose and mannitol with production of acid and gas but failed to ferment sucrose and gave positive methyl red, and negative Voges-Proskauer tests.

K. pneumoniae.—All strains retained their morphologic and colonial characteristics after the subcultures with or without the antibiotics. All of them were agglutinated by type A Friedlander's bacillus antiserum (rabbit) but only the strains which had been subcultured without antibiotic retained the ability to produce capsular swelling with this serum. The strains which had been subcultured twenty-seven times without antibiotic fermented dextrose, lactose, sucrose and mannitol with production of acid and gas, gave negative indol and methyl red and positive Voges-Proskauer tests (biochemical reactions similar to those of *Aerobacter aerogenes*). The streptomycin and neomycin strains produced acid without gas with the same sugars; they were indol and Voges-Proskauer negative and methyl red positive.

Str. pyogenes.—Both strains, *Sh* and *C203*, retained their original morphologic, cultural, and serologic reactions after all of the series of transfers.

Str. mitis.—This strain also retained its original morphologic, cultural, and biochemical reaction in each of the series.

D. pneumoniae.—This strain retained its morphologic, cultural, and serologic reactions, and remained bile soluble throughout all of the transfers, with the exception of the organisms which had been transferred twenty-seven times in neomycin broth. The latter were agglutinated specifically by type 3 antipneumococcus rabbit serum, but failed to show capsular swelling.

In summary, therefore, except for the deviations in the biochemical reactions of the Klebsiella strains, there were no major changes in morphology, serology or biochemical properties of the strains resulting from the repeated subcultures.

DISCUSSION

Although the original paper of Waksman and Lechevalier[5] described neomycin as a new antibiotic against streptomycin-resistant bacteria, only a limited number of observations had been made on the cross-resistance between these two antibiotics before the present study was undertaken. The studies which had been reported indicated that such cross-resistance did not develop.[6-8] Observations reported more recently suggested that cross-resistance occurs only in one direction. The Demerecs[9] noted that strains of *E. coli* resistant to streptomycin are sensitive to neomycin whereas strains resistant to neomycin are slightly more resistant to streptomycin than their parent strain. Gezon and Fasan[10] found essentially the same to be true for seven strains of group A hemolytic streptococcus which they studied.

In the present study, higher degrees of cross-resistance have been induced in some strains. It was shown that some organisms may develop moderate increases in resistance to neomycin as a result of repeated exposures to streptomycin and, confirming the previous observations of Gocke and Finland,[1] quite high degrees of resistance to streptomycin resulted from the acquisition of resistance to neomycin by subcultures in the latter antibiotic. As in the previous study, the development of resistance to neomycin was not accompanied by increased cross-resistance to antibiotics other than streptomycin. In the present study bacitracin and polymyxin B were included in addition to penicillin, aureomycin, terramycin, and chloramphenicol. The same was true with respect to the organisms which had been subcultured in streptomycin; except for the

cross-resistance to neomycin no increased resistance occurred to any of the other antibiotics studied. There was, indeed, an apparent increase in sensitivity of some of the strains to some of these other antibiotics following the repeated subcultures in either streptomycin or neomycin.

The difference in the rate of acquisition of resistance to the homologous antibiotic in broth and on agar is of interest. The transfers on streptomycin agar resulted in the development of resistant strains more rapidly and to a higher degree than with the corresponding transfers in broth. This is probably related to the size of the inoculum used in each of the successive transfers; in the case of the broth cultures these were in the order of 10^5 viable organisms per milliliter for the strains of *E. coli*, *K. pneumoniae*, and *Str. mitis* and 10^4 organisms per ml. for the strains of *Str. pyogenes* and *D. pneumoniae*, whereas the number of organisms in the loopful of culture used to inoculate each segment of the agar plates was estimated to be in the order of 10^6 or 10^7. No such difference was noted in the rate or degree of resistance to neomycin acquired by the two methods of subculture although the sizes of the inocula were of the same order of magnitude. This may be due to the fundamental difference in the resistance patterns of these two antibiotics as indicated by the Demerecs.[9]

However, with the relatively crude methods used in the present study, some of the organisms appeared to develop resistance to neomycin at about the same rate and to about the same degree as to streptomycin; and sometimes even to a greater degree. Whether this is due to the technique used or whether neomycin, which is said to be a mixture of antibiotics[11] may contain a component which behaves like streptomycin with respect to resistance, is not clear.

Because of the similar antibacterial spectra of streptomycin and neomycin, and the cross-resistance demonstrated here, the studies on the streptomycin-dependent variant were of interest. Neomycin could not replace streptomycin as an essential nutrilite for the streptomycin-dependent variant of *K. pneumoniae*. In view of the greater cross-resistance to streptomycin which results from the exposures to neomycin, it would have been of greater interest to see whether streptomycin could serve in such a manner to replace neomycin in the growth of a neomycin-dependent strain. Such a strain, however, has not yet been developed.

SUMMARY AND CONCLUSIONS

Serial subcultures were carried out in parallel in media containing streptomycin, neomycin or no antibiotic and the resulting bacterial strains were tested for sensitivity to both streptomycin and neomycin and also to six other antibiotics.

The organisms which had been transferred in antibiotic-free media retained their original sensitivity to both antibiotics.

The repeated subcultures in media containing streptomycin or neomycin resulted in increased resistance to the homologous antibiotic in most, but not every instance. The increase in resistance to streptomycin occurred more rapidly and to a higher degree when the subcultures were made in streptomycin agar than when they were done in streptomycin broth; the same was not true of the homologous resistance to neomycin.

The development of neomycin-resistance was quite regularly associated with a significant increase in resistance to streptomycin. The reverse did not occur so regularly, that is, neomycin resistance developed irregularly and was of relatively low degree in the organism which had become streptomycin resistant.

None of the strains which had been transferred in streptomycin or neomycin were more resistant to penicillin, aureomycin, terramycin, chloramphenicol, bacitracin or polymyxin B when compared with the corresponding strains that had been subcultured in antibiotic-free media. Some of the organisms, however, appeared to have become somewhat more sensitive to one or more of these 6 antibiotics following the subculture in streptomycin or neomycin.

A streptomycin-dependent variant of a strain of *Klebsiella pneumoniae* was developed in the course of this study. Neomycin could not replace streptomycin, in whole or in part, as an essential nutrilite for this streptomycin-dependent strain.

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THE HISTOCHEMISTRY OF LIPID CARBONYL COMPOUNDS

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INTRODUCTION

IN 1939 and 1940, Bennett[1, 2] described his phenylhydrazone reaction in the adrenal cortex and attributed it to the presence of ketosteroids "since no aldehydes with the solubility properties of the corticosterones have been detected in the adrenal cortex." Bennett apparently was not familiar with the work of the Feulgen group[3-6] on acetalphosphatides. Gomori[7] called attention to the fact that the pattern of distribution of the plasmal and phenylhydrazone reactions was identical, and concluded that the two reactions probably demonstrate the same kind of fatty aldehyde, presumably plasmal. Although much additional evidence has been brought forward to support Gomori's thesis,[8-15] a group of workers have continued to publish papers on the histochemical demonstration of ketosteroids by carbonyl reagents.[16-37] The attitude taken by these workers toward the criticism of Gomori and others can be summarized as follows: the compounds in question are soluble in acetone; they show sudanophilia, birefringence, fluorescence, and positive carbonyl tests. While no one of the tests is specific for ketosteroids, no single class of substances other than ketosteroids exhibits the entire battery of reactions. Moreover, positive reactions are obtained in all the endocrine glands which produce steroid hormones. For these reasons, the coexistence of these reactions is said to be indicative of the presence of ketosteroids.

Recently Camber[35] and Seligman and his group[36-42] have described a new reagent for carbonyl groups, the hydrazide of 2-hydroxy-3-naphthoic acid (Seligman and Ashbel's [SA] reagent) and asserted that positive reactions obtained with its use in frozen sections of formalin fixed tissues are due to the presence of ketones, and, in the case of the adrenal cortex, the corpus luteum and the interstitial cells of the testis, of ketosteroids. The authors did not assert that the reagent per se is specific for ketones, but based their statement on the following findings, considered indicative of the absence of lipid aldehydes from the tissues mentioned: (a) The Schiff reaction is negative.[36] (b) Treatment of the sections with mercuric chloride does not intensify the reaction; therefore plasmal can be ruled out. (c) Treatment of the sections with aniline for sixteen hours at pH 4 does not abolish the reaction although aniline is known to block aldehyde groups under these conditions. (d) Treatment of the sections with phenylhydrazine, followed by diazotized o-dianisidine (Blue B Salt), does not produce a blue formazan dye. (e) The Angeli-Rimini

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hydroxamic acid reaction is negative. (f) On studying the kinetics of the competitive removal of the hydrazide from carbonyl groups by o-sulfobenzaldehyde, they found that the hydrazide is rapidly detached from ketones but very slowly from aldehydes. The reacting material in the tissues releases the hydrazide at a rate characteristic of ketones rather than aldehydes.

This evidence presented in favor of the nonaldehydic nature of the carbonyl groups does not appear to be convincing. First of all, the statement about the negative Schiff reaction in the tissues examined is at variance with the findings of a number of workers who reported intensely positive Schiff reactions in the brain and in ketosteroid-producing tissues.[8, 9, 20, 21, 24-30, 43] Second the Angeli-Rimini reaction[44, 45] is not entirely dependable;[46] it is negative with several aldehydes,[47] and may be positive with ketones.[48]

Since the results of preliminary experiments have cast considerable doubt on the validity of the remaining points of evidence it was decided to reexamine critically the problem of lipid aldehydes vs. ketones in the tissues. It need not be emphasized that the demonstrability of ketosteroids in sections is a question of considerable importance.

EXPERIMENTAL

I. *Test Tube Experiments.*—

A. *Formation of formazans from various aldehydes and ketones:* It was found that not all aldehydes will give formazans. The phenyl-hydrazone of p-dimethylaminobenzaldehyde, for instance, produced no dye with Blue B Salt. On the other hand, dark red, alcohol-soluble dyes of unidentified nature were obtained from the phenylhydrazones of pyruvic and ketoglutamic acids. It is suggested that azo-coupling may have occurred into the active methylene group adjacent to the ketone group.

B. *The Schiff reaction of ketosteroids:* No reaction was obtained in the test tube with testosterone, desoxycorticosterone acetate (DOCA), and estrone. This is contrary to the findings of Dempsey and Wislocki,[19, 22] and in agreement with those of Albert and Leblond,[9] Oster and Oster,[49] and Ashbel and Seligman.[36]

C. *The solubility of ketosteroids in 50 per cent alcohol:* Saturated solutions of testosterone, DOCA, and estrone were evaporated, and the residue weighed. The solubility of testosterone was found to be 7.8 mg. per milliliter; that of DOCA, 5.8 mg. per milliliter, and that of estrone, 1.2 mg. per milliliter. It is very likely that the few micrograms or less of ketosteroid expected to be present in a tissue section would be washed out by the great excess of 50 per cent alcohol which is the vehicle of SA reagent, unless the reaction was very prompt.

D. *The rate of the SA reaction:* Equal volumes of 0.1 per cent solutions of ketosteroids in 50 per cent alcohol and of SA reagent were mixed and allowed to stand at room temperature. The first signs of turbidity with testosterone and DOCA appeared after about fifty minutes, and precipitation was complete by the end of two to three hours. Estrone did not produce a precipitate even after twenty-four hours. These facts, together with the relatively high solubility of the ketosteroids in 50 per cent alcohol, make it very likely that the ketosteroids would be dissolved much before the localizing reaction could take place. Precipitation of the ketosteroid would occur in the ambient medium rather than in the tissue, and the precipitate would settle at random.

II. *Model Experiments.*—

A. *Filter paper experiments:* Strips of filter paper were moistened with (a) 10 per cent solutions of ketosteroids and of a number of cholesterol derivatives (cholesterol, 7-hydroxy cholesterol, 7-ketocholesterol) in chloroform; (b) with 10 per cent solutions of linseed oil, olive oil, lard and tricaprylin in chloroform; (c) with chloroform containing both ketosteroids and lard. The strips were allowed to dry and were kept exposed to diffuse daylight for 30 days.

1. *Fluorescence:* The strips were examined in the filtered light of an ultraviolet light source daily. For about two days no fluorescence was noticed in any of the strips. On the third day, linseed oil started to show a faint bluish white fluorescence which rapidly increased in intensity and reached an extreme brilliance in about 7 to 9 days. Olive oil started to fluoresce on the fifth day, lard on the eighth day, and both reached a fairly high degree of fluorescence by the end of two weeks. Tricaprylin and the plain steroids never became grossly fluorescent. Steroid-lard mixtures behaved like plain lard. From these experiments it would appear that fluorescence is a property of auto-oxidation products of unsaturated fats rather than of ketosteroids.

2. *Carbonyl reactions:* A fairly intense Schiff and SA reaction was obtained with linseed oil immediately after drying, and a much less intense one with olive oil. The other strips showed no reaction either directly or after exposure to 10 per cent formalin for sixteen hours. As time went on, the reactions increased in intensity. After about one week, both linseed oil and olive oil reacted very intensely, lard moderately intensely, while the other substances showed no reaction at all or a very faint, barely noticeable one. Steroid-lard mixtures behaved exactly like plain lard. The Schiff and SA reactions invariably ran parallel. Results obtained with dinitrophenylhydrazine were quite similar to those of the Schiff and SA reactions, except for the much paler shade which made the evaluation of weak reactions difficult.

The Schiff reaction of all substances was blocked more effectively than the SA reaction by all carbonyl reagents tried (aniline, cyanide, hydroxylamine, and phenylhydrazine). The differences in efficiency were quite varied and seemed to follow no definite pattern in respect to either the reagent or the substance blocked.

Carbonyl reactions, just as fluorescence, appear to be due to products of auto-oxidation rather than to the presence of steroids. Special attention is called to the fact that the steroids, either plain or mixed with lard, gave no reaction either directly or after formalin treatment.

B. *Coujard*[50] *slides:* Lard, linseed oil, olive oil, and solutions of ketosteroids in melted lard were emulsified in warm 3 to 5 per cent gelatin (in a Potter-Elvehjem glass homogenizer), and marks were made with the emulsions on glass slides. The steroids (about 5 per cent) were agitated with melted lard for a few minutes, and both the solution and the undissolved excess of steroid were homogenized in gelatin. In this way some of the particles had a high, others a much lower steroid content. The slides were fixed in formalin vapor for twenty-four hours and stored in distilled water. Schiff and SA reactions were performed with them at intervals. The results were an exact duplication of those of the filter paper experiments.

III. *Experiments With Tissues.*—

A. *Frozen sections:* The following types of tissue were used: dog adrenal and testis; human adrenal, spinal cord, atheromatous aorta, cutaneous xanthoma, hypernephroma, necrotic carcinomas of the stomach and lung (one of each), and caseo-calcareous tuberculosis of the lung. The tissues were fixed in various mixtures (formalin, Bouin's fluid, saturated solution of picric acid in 50 per cent alcohol) for various lengths of time (one to sixty days; some tissues for over one year). Frozen sections were thoroughly washed in distilled water and stained with Schiff's and the SA reagent. Acetone-extracted sections were used as controls. The observations were as follows:

(a) Contrary to the statements of the Seligman group, formalin fixation is not essential for positive carbonyl reactions. Tissues fixed in 50 per cent alcoholic picric acid were only faintly positive immediately after twenty-four hours' fixation but became intensely positive by both the Schiff and the SA method after two days' soaking of the sections in water. By this time the pattern and the intensity of the reactions were indistinguishable from those obtained after formalin fixation.

(b) In agreement with the findings of most workers, and contrary to the statement of Seligman and Ashbel, an intense Schiff reaction was obtained in every single tissue in which the SA reaction was positive. Moreover, the patterns of distribution of the two reactions were very closely similar, often identical. In most tissues the differences were minor and changed in a rather erratic way, even within the same block of tissue, on exposure of the sections to air.

As a general rule, tissues fixed briefly tended to stain stronger with the SA reagent than with Schiff's, while after very long fixation usually, but not always, the reverse was true. In atheromata, the two reactions often showed a different pattern of distribution in that some groups of lipid droplets stained with both reagents, others only with Schiff's or SA's.

The intensity of the two reactions ran parallel. The only exception was the white matter of the brain which invariably stained much darker by the method of SA than by Schiff's.

(c) Intense carbonyl reactions (both SA and Schiff) were obtained not only in the adrenal, testis, and cord, but also in lipid droplets of atheroma, xanthoma, hypernephroma, necrotic tumors, and tubercles.

(d) Both the Schiff and SA reactions gradually increased in extent and intensity on prolonged exposure to formalin or water (and, presumably, to air), and a maximum effect was usually obtained after about fourteen days. The difference between sections exposed for two and for fourteen days was quite conspicuous. After two days, the staining of the adrenal cortex was often zonal or patchy, while after fourteen days it had spread considerably, often to involve not only all layers of the cortex, but also the periadrenal fat. As mentioned previously, the intensity of the SA reaction increased more rapidly than that of Schiff's, but the result after fourteen days was much the same. In the case of Bouin's fluid, the Schiff reaction was practically negative after four to six days of fixation, while the SA reaction was relatively strong. However, after soaking the sections in water for a few days, both reactions became quite intense.

(e) *Blocking experiments:* Sections were treated for sixteen to twenty-four hours at 37° C., and at pH 4 to 5 (except in the case of cyanide; see Table I) with a number of reagents known to block carbonyl groups, washed and subsequently stained with Schiff's and SA reagents. The results were surprising, and so varied as to render description very difficult. While the Schiff reaction was affected only quantitatively, the effects on the SA reaction were of two distinctly different types: (1) simple inhibition, and (2) a change in the color of the reaction from blue to purple-red or brownish-red. A tentative explanation of this phenomenon will be given later. The observations are summarized in Table I which is self-explanatory. It must be mentioned, however, that variations among individual specimens of the same type of tissue (for instance, human adrenal) were quite marked and appeared to depend mainly on the length of previous exposure of the tissue sections to air. This variability of results may explain such contradictory statements as those of Gérard[51] and of Verne[52] on the blocking of the plasmal reaction by dimedon.

TABLE I. EFFECT OF BLOCKING AGENTS ON THE SCHIFF AND SA REACTIONS IN FROZEN SECTIONS

TISSUE	REACTION	ANILINE 0.2 PER CENT	NaCN, 1 PER CENT pH 6.5	DIMEDON 0.5 PER CENT IN 50 PER CENT ALCOHOL	NH_2OH 5 PER CENT	PHENYLHYDRAZINE 2 PER CENT	SEMICARBAZIDE 5 PER CENT
Adrenal cortex	Schiff	3	1 to 2	2 to 3	3	3	0 to 1
	SA	1 to 2 X*	1 to 2	1 to 2 X	3	3 X	1 to 2
Periadrenal fat	Schiff	0 to 2	1 to 2	0 to 1	3	3	0
	SA	1 to 2 X*	1 to 2	1 to 2 X	3	3 X	0 to 1
White matter of spinal cord	Schiff	3	3	3	3	3	1 to 2
	SA	1 to 2 X*	2 to 3	2 X	3	3 X	1 to 2

Explanation of symbols: 0, no inhibition; 1, moderate inhibition; 2, marked inhibition; 3, nearly complete or complete inhibition; X, change in color.

*Highly variable.

The data given in the table show the relatively constant results seen in tissues after more than fourteen days' fixation or soaking in water. Only three types of tissue are listed because the lipids of the interstitial cells of the testis, of atheroma, xanthoma, hypernephroma, necrotic tumors, and tubercles behaved exactly as those of the adrenal cortex.

B. *Paraffin sections* of alcohol-fixed tissues (liver, stomach and intestine, salivary glands, etc.) of mice and rats were treated with 0.25 per cent periodic acid for ten minutes and washed under the tap. This treatment converted polysaccharides such as glycogen and mucin into aldehydes.[53] The slides were used, as carbonyl models of unquestionably aldehydic nature, to check the validity of Seligman's criteria for distinguishing between aldehydes and ketones.

Observations.—(a) In control sections, treated with periodic acid only, glycogen and mucin stained very deeply with both the SA and Schiff reagents. (b) Treatment of the sections with phenylhydrazine (six hours at 37° C., pH 4), followed by Blue B Salt in aqueous pyridine, produced some red forazan dye in gastric and intestinal mucus, much less dye in the mucus of the salivary gland and only the barest traces or none at all in glycogen. (c) The Angeli-Rimini reaction was invariably negative in all tissues. (d) SA reagent was removed by o-sulfobenzaldehyde from all tissues at a fairly fast rate. A definite fading of the shade of the final reaction became apparent within twenty minutes (at room temperatures), and by the end of two hours the intensity of the reaction was reduced to a fraction (probably less than 20 per cent) of the original.

(e) Blocking experiments were performed in essentially the same way as in the case of frozen sections, except that the time of exposure to the blocking agents was reduced to six to eight hours. The results differed greatly from those seen in frozen sections. First of all, the Schiff and SA reactions were affected in an identical fashion, regardless of the nature of the blocking agent. Second, only cyanide and hydroxylamine blocked both reactions completely. Phenylhydrazine blocked the reactions everywhere except in the salivary gland of the mouse where the intensity of the staining of mucus was only moderately reduced. After aniline and semicarbazide, a residual reaction of fair intensity persisted especially in mucus, and a faint reaction in glycogen. p-Aminobenzoic acid[54] blocked the reactions very incompletely; dimedon and sulfanilic acid[54] had very little effect.

DISCUSSION

The following conclusions can be drawn from the experimental data presented:

1. Fluorescence of lipid substances in the tissues is probably due to products of auto-oxidation and not to the presence of ketosteroids.

2. It is impossible to differentiate between aldehydes and ketones by Seligman's criteria. The Angeli-Rimini reaction can be negative with obviously aldehydic substances; the formazan test was negative with periodate-treated glycogen; the rate of removal of SA reagent from unquestionably aldehydic substances by o-sulfobenzaldehyde may suggest the presence of ketones rather than of aldehydes. The effect of blocking agents appears to depend primarily on factors other than the aldehydic or ketonic nature of the carbonyl group. Steric configuration and solubility are very likely to be the decisive factors. The reactivity of aldehydes and ketones is markedly influenced by the radicals attached to the carbonyl group;[55, 56] in the great majority of instances the reactions are not quantitative. The lipid-insolubility of semicarbazide may explain why it is such a poor blocking agent of lipid carbonyl groups while it is relatively efficient in the case of polysaccharide aldehydes.

Even within groups of lipids, solubility properties may vary considerably with individual substances (for instance, solubility of neutral fats and cholesterids but insolubility of phospholipids in acetone; progressive lipid-insolubility of unsaturated lipids on exposure to air).[57] Different solubilities may manifest themselves in different staining reactions. To mention only a few instances: Sudan III stains neutral fats intensely but phospholipids poorly. On the other hand, Sudan Black B stains neutral fats and phospholipids equally well. In the course of experiments with new fat dyes we found that Oil Blue CE (Ciba) stains myelin sheaths and the lipids of the adrenal cortex fairly intensely but leaves the periadrenal fat completely unstained, provided the tissue has not been exposed to formalin or water for longer than three to four days.

The reddish staining of lipids by SA reagent after aniline, dimedon or phenylhydrazine treatment was mentioned previously. In typical cases, the color after aniline treatment duplicates that of the dye obtained by azo-coupling SA reagent with Blue B Salt; in the case of dimedon and phenylhydrazine it is a more rusty brown. It is possible that after the blocking reactions have taken place, the resulting Schiff's base (or phenylhydrazone or dimedon derivative, respectively) has an increased solvent power for SA reagent. The latter will "stain" them, resist differentiation in 50 per cent alcohol and give a purple-red azo-dye in the last step. In the case of phenylhydrazine, the light brownish shade of the hydrazone is superimposed on that of the azo-dye. In the case of dimedon the possibility of azo-coupling into the dimedon molecule must be borne in mind (dimedon yields a dark red dye with Blue B Salt).

It is generally admitted that carbonyl reagents cannot differentiate sharply between aldehydes and ketones. However, Schiff's reagent[58] and dimedon[53] are considered to be much more specific for aldehydes than for ketones. The uniform results obtained with these reagents definitely support the assumption that the large bulk of lipid carbonyl compounds is of aldehydic character. Naturally, the admixture of a relatively small amount of ketones cannot be ruled out.

3. Intense lipid carbonyl reactions are obtained at all sites where cholesterol can be demonstrated, regardless of whether the tissue does or does not produce ketosteroids.

4. The three ketosteroids tried did not react at all with the SA reagent under the conditions of the histochemical experiment. Whether or not they do react with phenylhydrazine could not be determined. The shade of the phenylhydrazones and dinitrophenylhydrazones is far too pale to permit the detection of such small amounts of ketosteroids as can be expected to occur in tissues.

If ketosteroids cannot be demonstrated by histochemical methods at present available, and if the carbonyl reactions are due to aldehydes, the next question is, what sort of aldehydes must be considered and what is their source.

The first lipid aldehyde demonstrated in tissues was plasmal.[3] Plasmal is a mixture of plamitic and stearic aldehydes (possibly with a trace of unsaturated aldehydes), liberated from an acetalphosphatide precursor (plasmalogen) by mercuric chloride. The amount of plasmal in a given tissue is indicated by the difference in the extent and intensity of the Schiff reaction after and before treatment of the tissue with $HgCl_2$. This difference is very marked in the case of fresh tissues, but rapidly decreases on prolonged formalin fixation.[60, 61] It should be remarked that for a while both the difference and the absolute intensity of the reaction decline on storage. It is possible that plasmalogen is converted into nonreactive compounds, the nature of which is not clear. Very soon, however, there is a gradual increase in the extent and intensity of the blank reaction (i.e., without $HgCl_2$ treatment), the distribution of which is not entirely identical with that of plasmal. The large-scale formation of aldehydes from unsaturated fats by auto-oxidation is a well-known phenomenon, and its complicated intermediate phases have been studied extensively mainly by industrial chemists.[62-65] Cain[60, 61] and Hayes[66] were the first to call attention to nonplasmal lipid aldehydes in the tissues, although already Verne[67] and Gérard[51] had suspected the importance of oxidative processes in the genesis of lipid aldehydes. Judging from the intensity of aldehyde reactions in tissues which have been stored in formalin or water, aldehydes produced by oxidation (''rancid aldehydes'') are quantitatively more important than those deriving from acetalphosphatides.

All lipids of the animal body contain a certain percentage of unsaturated fatty acids. Esters of cholesterol and phospholipids excel in their degree of unsaturation. Kelsey and Longenecker[68] have shown that the percentage of unsaturated fatty acids in the cholesterol esters of beef plasma is twice, and the amount of linoleic acid four times, as high as in the glyceride fraction. Verne, Boulin, and Nepveux[69] have reported a high degree of unsaturation of fatty acids isolated from atheromatous lesions. Phospholipids of brain[70] and liver 71 have iodine numbers 100 per cent higher than depot fat. According to Rieckehoff, Holman, and Burr,[72] the fatty acids of heart muscle contain over ten times more triene acids than those of skeletal muscle. The distribution of unsaturated fatty acids is in excellent agreement with the intensity and extent of carbonyl reactions found in various tissues. The discrepancies between the patterns of the Schiff and SA reactions may be explainable by the different properties of the aldehydes produced from the various unsaturated acids (oleic, linoleic, linolenic, arachidonic, etc.).

Besides fatty acids, a few other potential sources of lipid carbonyl compounds have to be considered. The presence of unsaturated alcohols such as oleyl[73] and selachyl[74] alcohols in cetacean oils has been known for some time. Squalene, a potential precursor of cholesterol[75] occurs in human sebum in significant amounts.[76-78] In model experiments, squalene showed a white fluorescence and carbonyl reactions, rapidly increasing in intensity on exposure to air. This is in agreement with the findings of Farmer and Sutton[79] on the spontaneous peroxidation of squalene. Squalene-like hydrocarbons have been found in other tissues.[80]

The length of the initial period during which carbonyl reactions are negative depends not only on the chemical nature of the unsaturated fat itself, but also on the amount of carotenoids present in it. Carotenoids are known to protect fats from auto-oxidation[61, 81] until they themselves are destroyed by it.

On the basis of the data enumerated, it would appear that most if not all of the lipid carbonyl material occurring in animal tissues is of aldehydic nature and derives from the auto-oxidation of unsaturated compounds, mainly fatty acids. The "battery of reactions" asserted to be specific of ketosteroids is probably due to unsaturated fatty acid esters of cholesterol. Cholesteryl linoleate, for instance, would, after a brief exposure to air, give every single one of the reactions. The very intensity of carbonyl reactions as they are observed in the tissues suggests their being due to compounds which constitute a substantial percentage of the lipid material.

The last point to be considered is the alkali-solubility of certain lipid carbonyl compounds. Ashbel, Cohen, and Seligman[41] find that in the Sertoli cells of the testis the carbonyl reaction becomes negative after extraction of the section with a 0.5 per cent solution of sodium carbonate. They believe that the extracted fraction is phenolic (estrone). Since estrone does not give any histochemical reactions of carbonyl groups, the explanation must be different. It is very likely that the alkali-soluble fraction consists of carbonyl compounds of acidic nature (aldehyde- or ketone-acids, phosphatidic acids).

SUMMARY

The bulk of lipid carbonyl compounds in the tissues is of aldehydic nature and derives from the auto-oxidation of unsaturated fats, mainly esters of cholesterol. The ketosteroids themselves cannot be demonstrated by histochemical methods at present available.

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LABORATORY METHODS

THE USE OF THE EMISSION SPECTROGRAPH FOR THE DETERMINATION OF SERUM GOLD

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THE use of gold salts[1, 2] in the treatment of patients with rheumatoid arthritis has stimulated research on analytical methods for the estimation of gold in serum. A survey of the literature[3-9] showed that the current methods of analysis were unsuited to our requirements for speed and simplicity. The emission spectrograph employing a rotating electrode (Fig. 1), described by Boyle and associates,[10] was found to be highly satisfactory for the estimation of gold levels. The distinctive feature of this method is that dilution of the serum with 1 ml. of alkaline internal standard is all that is necessary to prepare the sample for analysis. The time required for a single estimation of gold in serum is reduced from a lengthy procedure to thirty minutes. Multiple samples may be analyzed with great rapidity and satisfactory accuracy, as is shown in Table I.

Equipment.—Applied Research Laboratories 1.5 meter grating spectrograph; A.C. spark source; Universal arc-spark stand; cylindrical lens; densitometer; rotating electrode assembly; spectrum analysis No. 1 film.

Instrumental Conditions.—2 kv-a; primary voltage 90; induction 8; cylindrical lens 100 per cent transmission; spark time 120 seconds.

TABLE I

SAMPLE (4 ML.)	GOLD ADDED (GAMMAS)	GOLD FOUND (GAMMAS)	PER CENT RECOVERED
Au-free pooled serum	4.8	4.8	100.0
Au-free pooled serum	9.6	9.6	100.0
Au-free pooled serum	14.4	16.2	112.4
Au-free pooled serum	19.2	20.0	104.0
Au-free pooled serum	12.0	11.5	95.8
Au-free pooled serum	24.0	22.0	91.8
Au-free pooled serum	36.0	36.3	100.8
Au-free pooled serum	48.0	43.0	89.7
Au-free pooled serum	60.0	62.2	103.6
Au-free pooled serum	18.1	18.7	103.2
Au-free pooled serum	4.8	5.5	114.5
Au-free pooled serum	9.6	8.6	89.5
Au-free pooled serum	14.4	13.0	90.4
Au-free pooled serum	19.2	18.0	93.8
Au-free pooled serum	4.8	5.3	110.2
Au-free pooled serum	9.6	9.5	98.8
Au-free pooled serum	14.4	14.7	102.2

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Reagents: 1. Internal standard solution: 8 Gm. of NaOH and 90 mg. of $Pb(NO_3)_2$ diluted to 1 liter. A fresh solution should be made every few days. 2. Stock gold solution: After reduction of $AuCl_3$ to free gold, using the hydroquinone method,[11] dissolve 1.0 Gm. in 25 ml. of aqua regia and dilute to 1 liter. 3. Working standard gold solution: Dilute 10 ml. of stock gold solution to 1 liter to obtain a final concentration of 10 γ per milliliter.

Procedure for calibration curves.—The calibration curve is prepared by the addition of known amounts of gold to normal serum, which is gold free. The alkaline internal standard is added to each sample, which is fortified with increasing amounts of gold.

Pipette 0.5, 1.0, 1.5, and 2.0 ml. of standard gold solution into 25 ml. beakers, containing 4.0 ml. of gold-free serum. Add exactly 1.0 ml. of the internal standard solution and mix thoroughly. Permit the samples to stand for twenty minutes, during which time the proteins hydrolyze sufficiently to prevent uneven coagulation on the rotating electrode. A

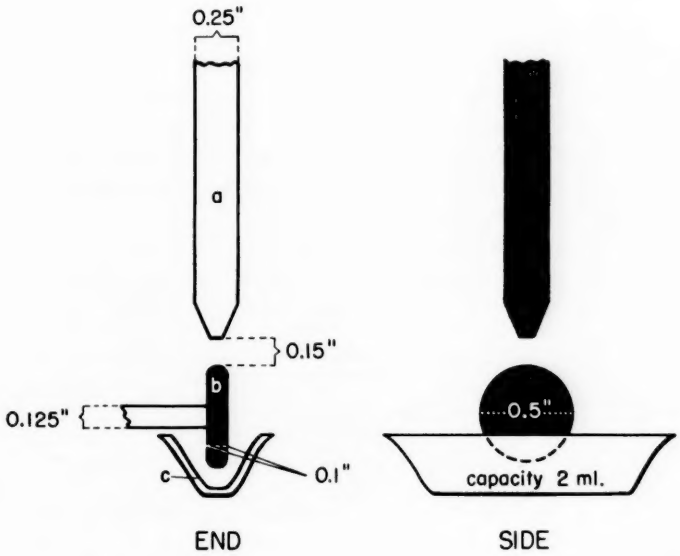

Fig. 1.

TABLE II

SAMPLE	DATE GOLD STARTED*	DATE OF SAMPLE	CUMULATIVE DOSES	SERUM (ML.)	AU FNG. (MG. %)
Patient A	10-23-50	12- 2-50	175 mg.	4	0.20
Patient A	—	12-14-50	225 mg.	4	0.22
Patient B	4-11-51	6-26-51	315 mg.	4	0.18
Patient B	—	7-19-51	360 mg.	4	0.20
Patient C	11-18-50	12- 7-50	85 mg.	4	0.24
Patient C	—	12-14-50	135 mg.	4	0.28
Patient D†	4-20-50	11-15-50	845 mg.	4	0.32
Patient D	6-21-50	6-21-51	845 mg.	4	0.09
Patient D	—	7-19-51	885 mg.	4	0.10
Patient E	5-31-51	6-26-51	85 mg.	4	0.23
Patient E	—	7-12-51	135 mg.	4	0.31
Patient F	4-12-51	7-12-51	520 mg.	4	0.20
Patient F	—	7-19-51	520 mg.	4	0.19

*Gold was given as myochrysin; it was started in a dose of 10 mg. and increased to a maximum of 25 mg. to 50 mg. at weekly intervals.

†Patient was on split dosage, taken off and then put back on after seven months without gold therapy.

portion of each sample is transferred to a 3.0 ml. porcelain combustion boat and adjusted on the spark stand (Fig. 1). Before immersing the electrode in the prepared sample, a dry pre-spark is made. With the lower electrode rotating and immersed in the sample, a second pre-spark of 120 seconds is made. The actual spectrogram is then made during the third 120-second sparking period. Ratios of gold to lead are plotted against gold concentrations to obtain the working curve for subsequent analyses. The wave lengths of the lines used are 2833.069 Å for the internal standard and 2675.95 Å for the gold.

RESULTS

The per cent recoveries which may be expected by adding known amounts of gold to gold-free serum are shown in Table I. Table II shows the amounts of gold analyzed in several serum samples from patients receiving gold therapy.

SUMMARY

This note describes a simple, rapid, and accurate spectrographic method for the estimation of gold in 4 ml. of serum. The spectrogram is obtained directly from the serum after dilution and incorporation of an alkaline lead internal standard, and the gold content is estimated by calibration against values obtained from the analysis of normal serum, after the addition of internal standard and known amounts of gold. The technique involved enhances the accuracy of the results, since the calibration curve and samples contain the same qualitative constituents. A single analysis may be accomplished in thirty minutes, while the averages for multiple analyses require no more than ten minutes per sample.

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DETERMINATION OF CEREBROSPINAL FLUID PROTEIN WITH THE FOLIN PHENOL REAGENT

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THE Folin phenol reagent[1, 2] when used with copper[3, 4] is a very convenient and exceedingly sensitive agent for the determination of protein. It has been used in recent years chiefly for the measurement of antigen antibody precipitates,[5, 6] but seems to be especially well suited to the measurement of the protein in cerebrospinal fluid.

Earlier the Folin reagent was used without copper.[7-13] As a result the color was weak and variable. The variability may help to explain why this reagent has not found wider clinical use. It is also true that the basic reactions have certain peculiarities which need to be considered in designing a reproducible method. Recently these peculiarities have been rather fully examined[14] and the method below results from this study.

METHOD

Reagents.—I. *Alkaline carbonate:* Dissolve 20 Gm. Na_2Co_3, and 0.5 Gm. sodium or potassium tartrate in a liter of 0.1N NaOH. II. *Copper sulfate:* 1 Gm. $CuSO_4\ 5H_2O$ per liter. III. *Working alkaline copper reagent:* Mix 45 ml. I. plus 5 ml. II. (Prepare daily.) IV. *Dilute Folin reagent:* Titrate 1 ml. of the reagent of Folin and Ciocalteu (Eimer and Amend, Fisher Scientific Co., New York) with 0.1N NaOH. (Add 50 ml. of water and titrate to a phenolphthalein end point.) On the basis of this titration dilute the commercial reagent so that 1 ml. is equivalent to 9 ml. of 0.1N NaOH. A dilution to about half strength will be required. The titration should be checked with new bottles of Folin reagent. V. *Standard protein:* Determine the protein content of a sample of normal human plasma by Kjeldahl or other standard procedure. Dilute with 0.85 per cent NaCl to give a 40 mg. per cent protein solution. This may be preserved with benzoic acid, but *thymol must not be used.*

Procedure.—Add 0.2 ml. spinal fluid to 10 ml. reagent III in a test tube or colorimeter tube. (Spinal fluid may be measured with an 0.2 ml. blood sugar pipette "to contain" and the pipette rinsed with the reagent.) Mix and allow to stand fifteen minutes. Add rapidly 1 ml. reagent IV mix *immediately and thoroughly,* allow to stand thirty minutes or longer (up to several hours) and read with any photoelectric colorimeter, using a red filter, e.g., the Klett photoelectric colorimeter with filter No. 66. (Absorption maximum is at λ 750 mμ.) Standards (0.2 ml. of the diluted plasma) and blanks are run at the same time as the spinal fluid. The colorimeter may be set to zero against the blank. Although the color is not quite proportional to the protein concentration[14] the deviation from linearity is not disturbing for routine purposes.

In calculating the spinal fluid protein concentration subtract 6 mg. per cent from the result to correct for the average color value of the nonprotein substances present (see below).

Rapid Bedside Method.—Add 2 drops of spinal fluid (measured with a standard dropper rather than the spinal needle) to 1 ml. 2 per cent Na_2CO_3 in 0.1N NaOH. Add one drop of 1 per cent $CuSO_4 \cdot 5H_2O$ in 2 per cent sodium acid tartrate and after five minutes one drop of *undiluted* Folin reagent (20 to 40 drops per ml.) and mix immediately. Compare after five

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minutes with a standard made from 1, 2, and 4 drops of a solution of 7 mg. per cent tyrosine in 0.1N HCl. These standards if measured with the same size drops as the spinal fluid will be equivalent to 25, 50, and 100 mg. per cent protein, respectively, in the spinal fluid. One set of standards may be used for a month without serious error if to each standard tube is added 3 drops or 0.1 ml. of 1M acetic acid and the tube is well stoppered.

RESULTS AND DISCUSSION

Two reactions are involved: (a) An initial interaction of the protein and copper in alkali, which appears to be related to the biuret reaction, and (b) a reduction of the phosphotungstic-phosphomolybdic acids of the reagent by both the copper-protein complex and by the tyrosine and tryptophane of the protein. At pH 10, the optimal pH for color development, the Folin reagent remains active for only a matter of seconds.[14] It is for this reason that final mixing must be very prompt and thorough.

Tyrosine and tryptophane give color in the absence of copper, but the rest of the protein gives no color without this metal. With plasma and spinal fluid proteins about 75 per cent of the color is dependent on copper. Proteins may differ considerably in chromogenicity (primarily because of differences in tyrosine and tryptophane content), but plasma and spinal fluid proteins are probably very nearly equivalent in this regard.

Some color is obtained with a trichloroacetic acid extract of spinal fluid. Fortunately, this color from nonprotein substances, calculated as protein, does not vary more than 2 mg. per cent from a mean value of 6 mg. per cent (Table I). The fact that this color is but little decreased by omission of copper indicates that it does not result from peptides or protein which escaped precipitation.

The proposed method gives results (Table II) which agree roughly with the usual turbidimetric sulfosalicylic acid method.[15] Because of known factors which can disturb turbidity measurements, and the specificity of the Folin copper procedure, the authors believe the colorimetric values more nearly represent the actual protein present. With 100 consecutive spinal fluids analyzed

TABLE I. PROTEIN VALUES FOR WHOLE CEREBROSPINAL FLUID COMPARED WITH APPARENT PROTEIN VALUES OF TRICHLOROACETIC ACID EXTRACTS

SAMPLE NO.	SPINAL* FLUID	TCA EXTRACT† WITH CU.	TCA EXTRACT† WITHOUT CU.	SAMPLE NO.	SPINAL* FLUID	TCA EXTRACT† WITH CU.	TCA EXTRACT† WITHOUT CU.
1	30.3	3.7	2.8	11	48.5	7.7	5.7
2	30.7	3.9	2.6	12	49.7	4.9	3.5
3	35.4	5.7	5.2	13	50.1	4.6	3.4
4	42.7	7.7	5.4	14	56.2	5.9	4.3
5	43.3	6.6	4.6	15	58.1	6.9	5.1
6	43.5	5.2	4.0	16	66.0	4.3	3.4
7	45.2	5.3	3.7	17	78.1	6.6	6.0
8	45.8	4.2	2.8	18	103.0	4.9	3.3
9	46.4	5.0	3.4	19	109.	6.3	5.5
10	46.9	7.1	6.0	20	261.	8.5	9.0
				Av.		5.8	4.5

All values calculated as milligrams of protein (or apparent protein) per 100 ml. spinal fluid.

*Protein values of the original spinal fluid analyzed by the proposed (Folin copper) procedure.

†Extracts prepared with 5 per cent trichloroacetic acid from each spinal fluid.

in a routine clinical laboratory using the Klett photocolorimeter the standard deviation of duplicate analyses was 3 mg. per cent protein. With a series of 20 spinal fluids analyzed with calibrated pipettes and using the Beckman spectrophotometer the standard deviation was 0.7 mg. per cent protein.

In our hands, therefore, the Folin method is more reproducible and is less liable to occasional serious error than the turbidimetric method. It may be noted that the Folin copper reaction is about 100 times more sensitive than the biuret reaction.

SUMMARY

The Folin phenol reagent used with copper has been adapted to the measurement of cerebrospinal fluid protein. The proposed method which requires only 0.2 ml. appears to have definite advantages in comparison with existing procedures. A rapid bedside method is also described which requires 2 drops of spinal fluid.

TABLE II. COMPARISON OF CEREBROSPINAL FLUID PROTEIN DETERMINED BY TURBIDIMETRIC AND FOLIN COPPER METHODS

SAMPLE NO.	TURBIDIMETRIC*	FOLIN	SAMPLE NO.	TURBIDIMETRIC*	FOLIN
1	11	15	7	28	37
2	17	15	8	29	41
3	18	19	9	34	38
4	21	23	10	39	54
5	26	37	11	62	71
6	26	30	Av.	28	35

All values mg. protein per 100 ml. spinal fluid.
*Method of Denis and Ayer.[15]

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A COLORIMETRIC METHOD FOR THE ESTIMATION OF POLYVINYLPYRROLIDONE (PVP) IN WHOLE BLOOD, PLASMA, AND URINE

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A SIMPLE colorimetric method was required for the estimation of polyvinylpyrrolidone (PVP) in blood, plasma, and urine in order to study rates of passage across capillary walls of PVP preparations of different average particle weights. A method suitable for amounts of PVP of 10 μg or more has been developed and is presented here. Results of the application of the method will be published later.

I. *Principle.*—

There is evidence that sulfonated azo dyes exist as aggregates in aqueous media under certain conditions of ionic strength and pH.[1] Changes in the state of aggregation are made evident by changes in optical density at a given wave length and by changes in the wave length of maximum absorption. PVP in aqueous solution combines with numerous substances including certain dyes[2]; it may bind individual dye molecules rather than aggregates of dyes. It was thought that the difference between the optical density of dye in water and the optical density of dye in water with PVP would be roughly proportional to the amount of PVP present. The absorption curves and the changes in these brought about by the addition of PVP were examined for a number of dyes under a variety of conditions. (Among the dyes tested were: Congo red, Trypan red, Benzopurpurin 4B, T-1824 [Evans' blue], Trypan blue, Niagara Sky blue 6B.) It was found that the red dye, Brilliant Vital red (Color Index 456) gave most promise. Adequate agreement was found between change in optical density at a given wave length and amount of PVP present. However, for small amounts of PVP, the estimation of the PVP concentration involved a small difference between two high optical density readings because of the presence of large amounts of uncombined dye. Subsequently, it was found that under conditions specified below most of the uncombined dye could be removed from the aqueous phase with *n*-butanol. The blanks were then reduced to very small values.

II. *Absorption Curves of Brilliant Vital Red (BVR) with and without Added PVP.*—

The curves shown in Fig. 1 were traced from recordings obtained on a Cary recording spectrophotometer with 1 cm. cuvettes. The solutions were made up as follows:

BVR solution	*BVR + PVP solution*
3.12 ml. H_2O	3.0 ml. H_2O
3.0 ml. M/1 phosphate buffer pH 7.0	3.0 ml. M/1 phosphate buffer pH 7.0
0.4 ml. 0.5 mg./ml. BVR	0.4 ml. 0.5 mg./ml. BVR
	0.12 ml. 3.5 per cent PVP

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This illustrates the shift in wave length of maximal absorption and the increase in optical density at a given wave length (e.g., $\lambda = 525$ mμ) on addition of PVP.

III. *Method.*—

1. *Reagents:*

a. *Protein precipitant:* (modified Somogyi)

I { 25 grams $ZnSO_4 \cdot 7 H_2O$ (reagent grade) } diluted to 1 liter with distilled water
{ 250 ml. 0.25N H_2SO_4 }

II 0.75N NaOH

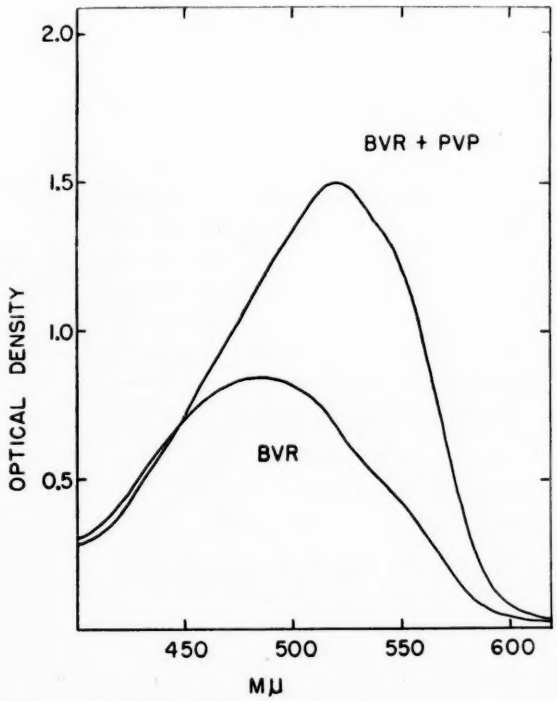

Fig. 1.—Absorption curves of Brilliant Vital Red solutions without (BVR) and with (BVR + PVP) added PVP.

25 ml. of solution I should be equivalent to 6.75 ml. (or slightly less) of the NaOH solution (phenolphthalein end point).

b. *Buffer:* M/1 phosphate buffer pH 7.0

0.676 mole K_2HPO_4 = 117.77 Gm.
0.324 mole KH_2PO_4 = 44.11 Gm. } diluted to 1 liter

c. *Dye:* Brilliant Vital Red (National Aniline)
0.5 mg./ml. in distilled water.

d. *n-Butanol:* Reagent grade.

2. *Procedure:*

a. *Removal of Proteins:*

1. *Plasma or serum*	2. *Whole blood*
0.25 ml. plasma or serum	0.20 ml. whole blood
1.25 ml. H_2O	1.00 ml. H_2O
0.60 ml. solution I	0.80 ml. solution I
0.15 ml. 0.75N NaOH	0.20 ml. 0.75N NaOH
Total volume 2.25 ml.	2.20 ml.

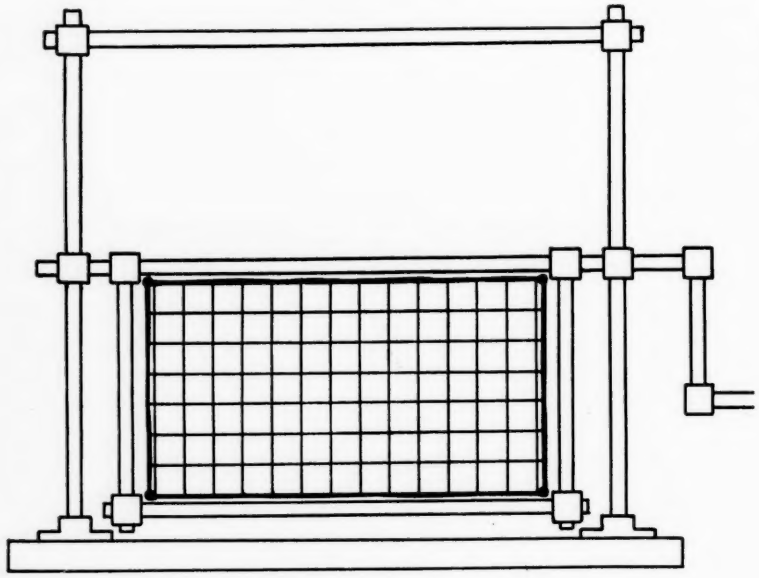

Fig. 2.—Rocker.

Allow to stand twenty minutes with occasional stirring with stirring rod. Centrifuge. The clear supernatants are removed.

b. *Reaction of dye with PVP:*

0.5 ml. supernatant	(or 1.0 ml. supernatant
1.0 ml. H_2O	and 0.5 ml. H_2O)
1.5 ml. M/1 phosphate buffer	(must be added before BVR)
0.2 ml. BVR	
3.2 ml. total volume	

Mix thoroughly; allow to stand forty-five minutes. Add 3.0 ml. *n*-butanol. Stopper with rubber stoppers; place in rocking device (Fig. 2). Rock gently for fifteen minutes.* Remove stoppers and cover tubes with parafilm. Centrifuge for thirty minutes at about 900 RCF. Pipette out lower layer (aqueous phase) into cuvettes, taking care not to carry over

*Motion should be no more violent than that which is used for mixing blood before taking samples for hematocrit estimations. A device such as is illustrated in Fig. 2 is necessary to ensure identical treatment of standards and unknowns. This particular device is made of Fisher Flexaframe parts. The wire test tube rack is held in place with rubber bands; a sheet metal plate of an area equal to that of the rack shown is placed over the tubes, and is also attached with rubber bands. The movement used is about 35 degrees away from the vertical rest position in each direction. The rocking should be sufficiently slow so that the liquid phases travel from one end of the tube to the other and so that emulsion formation is kept to a minimum.

any of the butanol phase. Some white precipitate may be found at the bottom of the tubes; this should not be disturbed in the pipetting. Seal cuvettes with parafilm to prevent evaporation of the butanol dissolved in the aqueous phase. Read optical density at the appropriate wave length. (Note: Wave length of maximum difference in optical density between blank and solution containing as high PVP: dye ratio as under II may be found to be different for different spectrophotometers or photocolorimeters. The wave length of maximal difference should be determined for each instrument. All data in this report have been obtained, unless otherwise specified, with 12 × 75 mm. cylindrical cuvettes at $\lambda = 510$ mμ with the Beckman B spectrophotometer at sensitivity setting 2.)

c. *PVP in urine:*

Urine, if free of protein, is treated as are the supernatants from blood and plasma samples. Volume of urine plus H_2O must be equal to 1.5 ml.

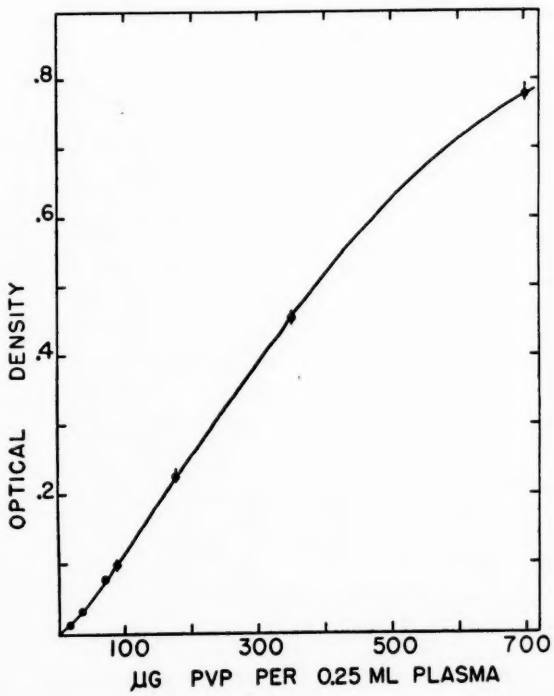

Fig. 3.—Optical density-PVP concentration curve for filtrates of plasma. To calculate the µg of PVP in each sample of filtrate analyzed divide the values for µg PVP per 0.25 ml. plasma by the factor 4.5.

d. *Standards:*

For plasma and whole blood known amounts of PVP (from appropriate dilutions of the material injected) are added to samples of blank plasma or blank whole blood, preferably from the *same* patient or experimental animal, obtained before PVP was given. (It appears from a few experiments that this precaution may not be essential.) Total volumes of plasma plus H_2O and/or PVP solution should be equal to 1.5 ml.; for whole blood the total volume of blood plus H_2O and/or PVP solution should equal 1.20 ml. Standards are treated in exactly the same manner as the unknowns. (Volumes of plasma or of whole blood should be the same in standards and unknowns.)

For urine appropriate dilutions of the injection material are treated as under 2b.

e. *Standard curves:*

A typical standard curve is shown in Fig. 3. The points represent the average of four determinations (duplicates of supernatants of two sets of standards). The vertical lines indicate the spread of the results. It will be noted: (1) that the curve is not a straight line but is sigmoid, and (2) that the spread is greatest at the highest concentrations of PVP. The most useful range of the method is about 10 to 100 μg per sample (i.e., in a volume of 1.5 ml. of diluted supernatant or urine under 2b).

The assumption that the dye is aggregated in an aqueous medium and binds as individual molecules is being investigated further.

f. *Blanks:*

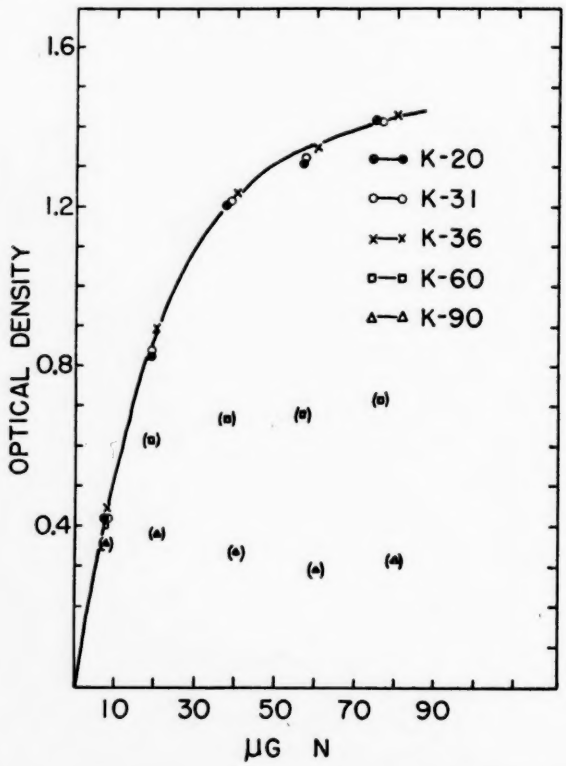

Fig. 4.—Optical density-PVP nitrogen data for PVP preparations with five different K values. The parentheses around certain points indicate that significant amounts of red precipitate had formed in these samples.

Unbound BVR is not completely removed from the aqueous phase by *n*-butanol. It is necessary, therefore, to subtract the optical density reading obtained with plasma filtrates containing no PVP from the optical density readings obtained with standards and unknowns. The mean of the optical density readings of a series of fifteen blanks, under the conditions described in this article, was 0.035, with a standard deviation of 0.003.

IV. *Applicability of Method to PVP Preparations of Different K Values.*—

None of the PVP preparations currently available is monodisperse; the polydispersity varies from one preparation to another. The preparations are characterized by their K values which are a function of the viscosity of the preparations relative to the viscosity of

water. The behavior of preparations of different K values* in the colorimetric method was examined. The results are shown in Fig. 4, where optical density is plotted against μg of nitrogen (determined by macro-Kjeldahl) for five preparations. There is no significant difference between the results with the preparations with K values of 20, 31, and 36. At the lowest concentration tested, the preparation with K = 60 also falls is line with the other results. At the higher concentrations with this preparation and at all concentrations tested of the preparation with K = 90, a red precipitate formed in the aqueous phase in amounts roughly proportional to the amount of PVP present. From the data, it appears that at very low concentrations of PVP the method may be applicable to the K = 90 preparation. Because the preparations to be used clinically will probably have but insignificant amounts of very high molecular weight particles, it is unlikely that the nonapplicability of the method to PVP preparations of high K values represents a serious limitation. Recovery from plasma of PVP preparations of K values 20, 31, and 36 appears to be complete; the standard curves do not differ significantly from one another or from standard curves in water provided the results are compared on the basis of nitrogen content.

DISCUSSION

A few rough tests indicate that probably the only critical feature of the procedure is the maintenance of the proportion of phosphate buffer to total volume under 2b. The use of other buffer systems than phosphate and other pH numbers than pH 7.0 ± 0.1 has not been investigated yet. If too little buffer is added extraction of uncombined dye is incomplete; if too much buffer is added nearly all the dye will go in the butanol phase. Standards must be run at the same time as unknowns. Finally, exactly the same amount of dye must be added to each tube of a series; delivery of the dye by micropipette is recommended. Applicability of the method to tissues has not been tested.

T-1824, in concentrations which usually obtain in plasma after the injection of dye for the estimation of plasma volume, does not interfere with the procedure for PVP: the T-1824 is precipitated with the proteins by the modified Somogyi reagents. T-1824 appears in the urine of dogs after the intravenous administration of PVP even in the absence of proteinuria; however, in the few experiments carried out in this laboratory, the renal clearance of T-1824 was about one-tenth that of the PVP. Under these circumstances, the relatively small amounts of T-1824 present in the urine do not cause any significant interference with the determination of PVP.

SUMMARY

A colorimetric method for the estimation of PVP in whole blood, plasma, and urine is presented. The method depends on the binding of the dye, Brilliant Vital Red, to PVP in aqueous solution and removal of unbound dye with *n*-butanol. The applicability and some limitations of the method are discussed.

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